INTERSPECIFIC-DERIVED AND JUVENILE RESISTANCE TO ANTHRACNOSE IN LENTIL

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ABSTRACT

Anthracnose, caused by *Colletotrichum truncatum*, is a major disease of lentil (*Lens culinaris* Medik.) on the Canadian prairies. Resistance to the more virulent race Ct0 of the pathogen is extremely rare within the *L. culinaris* gene pool thus resistance is being introgressed from *Lens ervoides*. The overall hypothesis of this project was that resistance derived from *Lens ervoides* accession L-01-827A would provide uniquely low levels of resistance to *Colletotrichum truncatum*. Individual studies were performed to: i) test field-resistance of interspecific lines; ii) develop a cutting-method to generate replicated phenotypic data on single plants; iii) study the deterioration of resistance between the juvenile phase (JP) and adult phases (AP) in the line 'CDC Redberry'; and iv) examine genetic control of resistance in a susceptible and partially resistant background. Field evaluations of *L. culinaris* x *L. ervoides* lines indicated resistance genes from the wild species will provide unprecedented levels of disease control and heritability of the trait was estimated to be moderate. Single plant phenotyping of resistance is unreliable, thus the feasibility of using clonal propagation of individual plants to generate replicated ratings was evaluated. Results showed consistency in segregation ratios between cutting and seedling-derived plants of the same population, thus the method was utilized for testing of genetic control of segregating populations. 'CDC Redberry' showed varying disease levels depending on the age of the plants with resistance acquisition in the JP that decreased as the plants proceeded through the AP. The F_1, F_2 and $F_{2:3}$ generations of two introgression populations were tested for resistance. Resistance from *L. ervoides* to both races of *C. truncatum* appeared to be due to the same gene(s) or from the same linkage block in both populations. Models for genetic control were consistent between F_2 and $F_{2:3}$ generations, however were different between the populations depending on whether the interspecific line was crossed into a susceptible or partially resistant *L. culinaris* background. However duplicate recessive epistasis seemed to control susceptibility in the susceptible background and when JP resistance was not a factor. There were significant differences between the JP and AP on more than a third of the F_2s tested supporting different resistance gene action based on growth phase. Resistance in the

JP seemed to be due to dominant and recessive epistasis. It was postulated that the the populations may have had segregation distortion commonly found in interspecific populations, thus the previously described genotypes for cotyledon colour and albino plants were used to test the hypothesis of segregation distortion. Segregation of these traits were found to be similarly distorted to previously reported interspecific *L. culinaris* x *L. ervoides* populations supporting the hypothesis that continued segregation distortion was found in the introgression populations. Overall, it was found that resistance derived from *L. ervoides* accession L-01-827A is a highly effective source for the lentil breeding program, however the result suggest more than one backcross to *L. culinaris* may be necessary to properly integrate the resistance genes to eventually obtain fully fertile, adapted lentil cultivars.

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DEDICATION

For those who have provided me with unconditional love and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AP	Adult Phase
CDC	Crop Development Centre
COS	Conserved Orthologous Sequence
EST	Expressed Sequence Tag
Expt	Experimental Repeat
GLM	General Linear Model
hpi	Hours Post Inoculation
ICARDA	International Center for Agricultural Research in the Dry Areas
ITAP	Intron-Targeted Amplified Polymorphism
JP	Juvenile Phase
LL	Leaf Lesions
NSF	North Seed Farm
QTL	Quantitative Trait Loci
RAPD	Random Amplified Polymorphic DNA
RIL	Recombinant Inbred Lines
SDB	Shoot Die Back
SL	Stem Lesions
SNP	Single Nucleotide Polymorphism
SSR	Simple Sequence Repeat

CHAPTER 1

INTRODUCTION

Canadian production of lentil, *Lens culinaris* Medik., accounts for close to 30% of world production (Food and Agriculture Organization of the United Nations, 2008) and with increasing interest in the crop it is expected that disease will continue to be a major barrier to yield and quality optimization. Given warm, moist conditions, the disease anthracnose can cause significant yield losses on the Canadian prairies (Chongo and Bernier, 2000a and 2000b). Anthracnose is caused by the pathogen *Colletotrichum truncatum* (Schwein.) Andrus& W. D. Moore, and two races of *C. truncatum* have been identified (Buchwaldt et al., 2004). Race Ct1 is considered the less virulent race while Ct0 is more virulent. Resistance to Ct1 within the *L. culinaris* primary gene pool is abundant, however resistance to race Ct0 is limited.

Breeding for resistance in lentil to *C. truncatum* is of great importance for the Crop Development Centre (CDC), especially to the highly virulent race Ct0. Wild relatives have been a valuable source of resistance to many crop species (Hajjar and Hodgkin, 2007), thus resistance genes in the secondary gene pool of *L. culinaris* are being introgressed. Previously, a recombinant inbred line population of an interspecific cross between *L. culinaris* and an accession of *L. ervoides* was developed and it was shown that resistance was transferable from the wild relative. However, genetic control of the trait could not be resolved due to suspected segregation distortion (Fiala et al., 2009). This project was initiated to investigate genetic control of resistance derived from *L. ervoides* in a *L. culinaris* background. Prior to initiation of this study, partial resistance to race Ct0 was also identified in the accession VIR421 and a family of breeding lines (3155S) that was developed at the CDC.

1.1 Objectives

The overall objective of this project was to enhance understanding of the genetics governing resistance (from both *L. culinaris* and *L. ervoides*) to *C. truncatum* in lentil and to test the hypothesis that resistance derived from *L. ervoides* accession L-01-827A would provide unprecedented control of the pathogen. Three key issues associated with this pathosystem were address in the studies performed: i) the lack of resistance to *C. truncatum* race Ct0 in primary *L. culinaris* gene pool; ii) the unreliability of single plant phenotyping of resistance, and iii) the deterioration of resistance between the juvenile phase (JP) and adult phases (AP) in the line 'CDC Redberry'. Studies performed to address each of these included a multi-year field evaluation of *L. ervoides*-derived resistance; development and testing of a clonal propagation method to generate replicated disease data on an individual plant; experiments examining resistance at different plant ages to establish a sound phenotypic description of juvenile and adult-phased resistance; development and phenotyping of introgression populations for resistance at different phases and to both races of *C. truncatum*; as well as phenotyping a population for cotyledon colour and albino plants. Results and conclusions from this project will have an impact on the understanding of this pathosystem and how to best select for resistance with the lentil breeding program at the CDC.

CHAPTER 2

LITERATURE REVIEW

2.1 Lentil Domestication and Production

Lentil was one of the first domesticated plant species approximately 10 000 years ago along with einkorn, emmer, barley and pea (Harlan, 1992) in the foothills of the mountains of southern Turkey and northern Syria (Cubero et al., 2009). Barulina (1930) suggested the centre of origin of lentil was in the mountains of the Hindu-Kush and Himalayas based on diversity of characteristics found in endemic *Lens* populations but archaeological evidence better supports domestication in the Middle East along with other pulses and cereals (Ladizinsky, 1993). Cultivation has been documented through ancient Egyptian and Greek cultures to the middle ages and through the 19th to 21st centuries. Productivity in poor soils and harsh climatic conditions is the reason lentils have been maintained as a protein food crop throughout the ages (Cubero et al., 2009).

In the past three decades, the world-wide production of lentil has almost tripled with 3.5 Mt produced in 2008 (Food and Agriculture Organization of the United Nations, 2008). The crop is grown primarily in arid or semi-arid regions of Canada, India and other south Asian countries, Turkey, Australia, China and northern Africa. In Canada, there were more than 900 000 ha grown in 2009 producing almost 1.5 Mt (Saskatchewan Agriculture and Food, 2009). This accounted for 29% of world lentil production making Canada the largest producer and exporter of lentil world-wide (Food and Agriculture Organization of the United Nations, 2008). The province of Saskatchewan produces 99% of the Canadian lentil crop (Saskatchewan Pulse Growers, 2010). Currently, lentil is an important source of dietary nutrients in Afghanistan, Bangladesh, India, Nepal, Pakistan, Ethiopia, Morocco, Tunisia, Sudan, Iran, Syria, Turkey, Egypt and Iraq (Sarker and Erskine, 2006). Despite marketing

efforts to increase domestic consumption in developed countries, use of lentil is relatively limited in countries such as Canada, the USA and Australia (Muehlbauer et al., 2009). The market classes of lentils grown in Saskatchewan include both green and red. Large green 'Laird' types, medium and small greens and French greens are all consumed in the Mediterranean, Middle East and South America in soups. Red lentils are primarily consumed in South Asia in dhal-type dishes as well as in West Asia, North Africa, Mediterranean countries, Egypt and Turkey (Muehlbauer et al., 2009; Sarker and Erskine, 2006). From the 1980s to the 1990s, green lentils were predominantly grown, but since the 1990s red lentils have been increasing in acreage with that class occupying approximately half the Saskatchewan lentil production in recent years (Muehlbauer et al., 2009).

2.1.1 The Genus *Lens*

Lens Miller. is part of the tribe *Vicieae* (Family Leguminosae, Sub-family Papilionaceae) along with three other closely related genera, *Vicia* L. (faba bean), *Lathyrus* L. and *Pisum* L.(pea). There are many intermediate forms within the tribe with morphological continuums from one genus to the other (Ladizinsky, 1993), especially for *Vicia* and *Lens* (Cubero et al., 2009). This can be attributed to active evolution radiating from a single origin (Cubero et al., 2009).

The genus *Lens* also has frequent intermediate forms. *Lens ervoides* (Brign.) Grande, *L. nigricans* (M.Bieb.) Godr., *L. lamottei* Czefr., *L. odemensis* Ladiz., *L. tomentosus* Ladiz., *L. culinaris* ssp. *orientalis* (Boiss.) Ponert and *L. culinaris* Medik ssp. *culinaris* have been differentiated based on presence of toothed stipules, leaflets per leaf, rachis length, aristate peduncle or awn, calyx teeth / corolla ratio, peduncle / rachis ratio, pod pubescence and seed diameter (Cubero et al., 2009; Ferguson et al., 2000; Ladizinsky, 1993 and 1997). *Lens culinaris* ssp. *orientalis* is the wild ancestor to *L. culinaris*. Based on morphological and molecular markers, *L. culinaris* was proposed to have four subspecies, *culinaris, orientalis, tomentosus* and *odemensis* with *L. ervoides*, *L. nigricans* and *L. lamottei* being other species in the genus (Ferguson et al., 2000). However, hybridization within *Lens* indicates that morphological discrimination of the species is unclear (Ladizinsky, 1993).

Based on hybridization barriers, *L. orientalis* belongs to the primary gene pool, *L. odemensis* to the secondary and *L. nigricans and L. ervoides* in the tertiary (with possibility of being in the secondary with embryo rescue). *Lens tomentosus* and *L. lamottei* belong to either the secondary or tertiary gene pools (Cubero et al., 2009). Crosses between *L. culinaris* and *L. ervoides* result in abortion after the embryo reaches the heart stage. Embryos can be rescued and grafted to develop hybrids (Fiala et al., 2009). It has been found that producing hybrids of the two species is more difficult with *L. ervoides* accessions from Ethiopia compared with *L. ervoides* accessions from Yugoslavia and Israel (Ladizinsky, 1993). It has been proposed that *L. ervoides* is a subspecies of *L. nigricans* (Ladizinsky et al., 1984) as the two species readily cross to produce vegetatively normal but sterile hybrids (Ladizinsky, 1993).

Wild lentils are primarily found in the Mediterranean basin and extend east up to Tajikistan (Ladizinsky, 1993). The species *L. ervoides* grows in the Mediterranean region (Israel, Syria, Turkey, Yugoslavia, Italy, Spain and Algeria) and unlike other *Lens* species, have isolated populations in Ethiopia and Uganda. In the Mediterranean region, plants often grow in shady habitats under a canopy of trees or among shrubs from sea level up to 1000 m in altitude. At high altitudes in Uganda and Ethiopia (2800 to 3000 m), *L. ervoides* grows in open habitats among perennial grasses. The wild progenitor of lentil, *L. orientalis* , is found from Turkey to Tajikistan and from Iran to the Crimean Peninsula growing in open or partially shaded habitats from 500 to 1700 m alongside other legumes or grasses (Ladizinsky, 1993).

2.2 Lentil Genetics and Breeding

Lentil is a diploid (2n=2x=14), self pollinating, annual crop with a genome size of 4063 Mbp / 1C (Arumuganathan and Earle, 1991). All *Lens* species have the same chromosome number and share karyotypes that have three pairs of metacentric or submetacentric chromosomes, three pairs of acrocentric chromosomes and a pair of metacentric chromosomes with secondary constrictions very close to the centromere (Ladizinsky, 1993).

2.2.1 Improvement and breeding

Lentil was an under-researched crop until the late 1970s when the International Center for Agricultural Research in the Dry Areas (ICARDA) initiated systematic lentil research. Currently, research programs related to germplasm development exist in over 40 countries, however are most substantial in India, Canada, Turkey, Iran, Australia, Nepal, Bangladesh, Syria, Ethiopia, Morocco, Pakistan and the USA (Sarker and Erskine, 2006). Improvement of lentils for developing countries focuses on higher yield, improved disease resistance, adaptation for mechanical harvesting and higher residue yields for animal fodder (Muehlbauer et al., 2009). Additionally, research at ICARDA for improved lentil production includes optimizing agronomic performance along with germplasm development for increased drought tolerance, tolerance to salinity and boron toxicity or boron-deficiency, increasing resistance to fusarium wilt, rust and ascochyta blight and broadening the overall genetic base of germplasm, especially for South Asia. An international testing program for lentil has been established by ICARDA for many of these traits at target sites to increase understanding of adaptation to various ecological niches and screen against local stresses (Sarker and Erskine, 2006). Developed countries have also benefited from ICARDA germplasm development as the material is routinely used to broaden the genetic base for traits under selection. Since ICARDA's initiation of lentil research, more than 100 cultivars that have been released can be traced back to ICARDA material (Muehlbauer et al., 2009). Furthermore, since 1980 the global lentil productivity has increased 58% (Sarker and Erskine, 2006) resulting in large production increases in areas of Turkey, South Asia, West Asia and North Africa.

The largest global collection of lentil germplasm is maintained at ICARDA which includes more than 11 000 *L. culinaris* landraces and wild *Lens* collections from South, West and Central Asian and North African countries. Germplasm in unexplored areas in Central Asia and the Caucasus continues to be added to the collection (Furman et al., 2009; Muehlbauer et al., 2009; Sarker and Erskine, 2006). There is documented variability in a large range of traits in the collection that are being utilized in breeding programs world-wide (Sarker and Erskine, 2006). A core collection of 1000 accessions was established from the collection and it is being

analyzed for molecular diversity (Ford et al., 2009). Other valuable collections of *Lens* germplasm include those from the Australian Temperate Field Crop Collection, Pullman United States Department of Agriculture Agricultural Research Services, the N.I. Vavilov All-Russian Research Institute of Plant Industry and the National Bureau of Plant Genetic Resources in India along with working collections of various breeding programs world-wide (Furman et al., 2009).

Multiple crop species, largely tomato, wheat, rice, potato, oat and sunflower, have been improved by introgression of genes from wild relatives and the use of wild relatives in crop improvement over a vast number of species is continuing to increase. Most wild genes have been introgressed to improve pest and disease resistance, but also for abiotic stress resistance, yield, quality traits and male sterility or fertility restoration (Hajjar and Hodgkin, 2007). Resistance to ascochyta blight in lentil has been enhanced by introgression of resistance genes from *L. odemensis* and *L. ervoides* (Ahmad et al., 1997) and variation for both drought tolerance and freezing tolerance has been identified in wild *Lens* collections (Hamdi et al., 1996; Hamdi and Erskine, 1996) however it does not appear there is incorporation of wild genes in any currently released lentil cultivars (Hajjar and Hodgkin, 2007). Recently, various wild *Lens* accessions were identified as being resistant to anthracnose and ascochyta (Tullu et al., 2006 and 2010) and resistance to anthracnose was found in an interspecific population between *L. culinaris* and *L. ervoides* (Fiala et al., 2009).

2.2.1.1 Lentil breeding objectives for Saskatchewan

The lentil breeding program at the Crop Development Centre (CDC) has four main focus areas for genetic improvement: i) resistance to fungal diseases; ii) adaptation and productivity; iii) agronomic traits; and iv) quality profiles. Resistance to ascochyta blight, caused by *Ascochyta lentis*, was a major objective in the late 1980s resulting in the release of resistant varieties in the late 1990s. Since the disease anthracnose was identified in the early 1990s, breeding for resistance in lentil has been of the utmost importance, especially to the highly virulent race Ct0. Resistance to minor diseases, such as grey mold (*Botrytis cinerea*), white mold (*Sclerotinia sclerotiorum),* stemphylium blight (*Stemphylium botryosum)* and fusarium root rot,

have been investigated since 2001 and it is expected routine screening for resistance to these diseases will be an integral part of the lentil breeding program in the future. Productivity is improved by incorporation of new germplasm into the breeding program followed by intensive re-selection for adaptation and use of a breeding system that mirrors agronomics practices of lentil growers. The agronomics of lentil have recently been improved by the availability of imidazolinone resistant lentil cultivars circumventing some weed control issues faced by growers. Lodging tolerance is now a major agronomic breeding objective. Green lentils are selected for uniformity of seed coats and colour retention whereas red lentils are selected for milling efficiency and uniform cotyledon colour. Both lentil types are selected for increased seed thickness and uniformity of diameter and thickness. Based on ongoing basic research into quality profiles related to biofortification, characteristics such as micronutrient bioavailability, carbohydrates, folates and proteins will be selected for in the future (Vandenberg, personal communication; Muehlbauer et al., 2009).

2.2.2 Molecular research in lentil

Currently, lentil is regarded as an orphan crop for gene discovery and functional genomic studies with only a fraction of the number of expressed sequence tags (ESTs) available compared to chickpea, cowpea and field pea (Ford et al., 2009). However, various projects are underway at the CDC and other institutes world-wide to generate mass quantities of sequence data for lentil.

2.2.2.1 Linkage maps

Until recently, linkage mapping efforts in lentil have been based on arbitrary molecular markers such as random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP) and inter simple sequence repeat markers (Durán et al., 2004; Eujayl et la., 1998a; Ford et al., 2009; Hamwieh et al., 2005; Kahraman et al., 2004; Rubeena et al., 2003). Durán et al. (2004) and Hamwieh et al. (2005) used arbitrary markers as well as simple sequence repeat (SSR) markers to generate intersubspecific and intraspecific maps, respectively. However, in total only 33 primer pairs for SSR polymorphisms in lentil have been developed, thus lentil

research groups at Washington State University USDA-ARS and ICARDA have generated new SSR markers using various methods (Ford et al., 2009).

Qualitative traits currently mapped in lentil include cotyledon colour, anthocyanin in the stem, seed coat pattern, pod dehiscence, ground colour of seed (Durán et al, 2004), fusarium wilt (Eujayl et al., 1998b), anthracnose resistance (Tullu et al., 2003) and seedling frost tolerance (Eujayl et al., 1999). Many agronomically important traits are quantitatively controlled and the quantitative trait loci (QTL) that have been found in lentil are for plant height and number of days to flower (Tullu et al., 2008), winter hardiness (Kahraman et al., 2004) and ascochyta blight resistance (Ford et al., 1999).

Despite efforts to generate molecular maps of lentil from various lentil research groups world-wide, the current routine application of markers to lentil breeding programs is limited. This is due to non-transferability of markers in different genetic backgrounds or across different labs, lack of close association of markers with traits of interest and cost and ease of implementation of screening techniques (Ford et al., 2009). For example, in-house markers flanking anthracnose and ascochyta resistance genes have been developed at the CDC (Tar'an et al., 2003), however are not currently used for routine screening in the lentil breeding program. Recent mapping efforts have been directed towards generating gene-specific and microsatellite markers that may aid the integration of marker assisted selection in breeding programs. The reproducibility and robustness of the new marker types is necessary in developing high-density genome linkage maps that are useful across genetic backgrounds (Ford et al., 2009).

The first gene-based linkage map of lentil was developed using intron-targeted amplified polymorphic (ITAP) markers developed from alignment of ESTs of *Medicago truncatula* and *Lupinus* spp. or *M. truncatula*, *Lupinus albus* and *Glycine max* (Phan et al., 2007). Out of 626 ITAP markers screened, only 98 produced polymorphic markers from which 79 were mapped onto the Australian population 'Digger' (ILL5722) x 'Northfield' (ILL5588). Additionally, 18 SSRs were mapped to integrate the map with that developed by Hamwieh et al. (2005). Seven linkage groups that were 80 to 275 cM long were found with five to 25 markers each, resulting

in a genome map of 928 cM. Comparisons with *M. truncatula* showed a macrosyntenic relationship between the two species with moderate chromosomal rearrangements (Phan et al., 2007), similar to what has been observed with other legumes species (Choi et al., 2004a and 2004b; Weeden et al., 1992). Identification of synteny with the model species *M. truncatula* will allow for identification of new markers linked to traits, candidate genes and will expedite the isolation of genes (Ford et al., 2009; Phan et al., 2007).

2.3 Anthracnose in Lentil

The disease anthracnose, caused by *Colletotrichum truncatum* (Schwein.) Andrus & W.D has been reported in lentil crops in Canada, North Dakota, Bulgaria, Pakistan, New Zealand, and is expected to be found in other lentil-producing countries (Kaiser et al., 2000). Anthracnose is a major disease of lentil in the prairies and can cause severe yield losses and reduction of seed quality. Yield losses on susceptible and resistant lines have been documented to be up to 57% and 28%, respectively, under high disease pressure when the disease is not controlled with fungicide (Chongo et al., 1999). Anthracnose was first identified in Manitoba in 1987 (Morrall, 1988) and was found in Saskatchewan in the growing season of 1990 (Morrall and Pedersen, 1991). Symptoms of anthracnose initially appear as superficial lesions on young stems and leaves and when the crop reaches the early flowering stage, premature leaflet abscission on lower leaflets occurs. Given adequate rainfall, conidia form in acervuli on stems and abscised leaflets, and are splash-dispersed to uninfected tissue which causes stem lesions to gradually move up the stem. Enlarging stem lesions can girdle stems causing plants to wilt and die (Buchwaldt et al., 1996). Disease infection is worst when there is excessive moisture in late summer that prolongs growth and delays harvest (Morrall et al., 2008). Survival of the pathogen is aided by the development of microsclerotia which remain in the field after harvest (Buchwaldt et al., 1996). Optimal temperature for disease development is 20 to 24°C and disease severity increases with higher temperatures (Chongo and Bernier, 2000a and 2000b).

The pathogen *C. truncatum* is classified as an ascomycete. To date, the teleomorph of *C. truncatum* , *Glomerella truncata* sp. nov., has not been found in the

field (Banniza, personal communication), but perithecia will form when induced under laboratory conditions (Armstrong-Cho and Banniza, 2006) similar to *Colletotrichum lindemuthianum* and *C. graminicola* (Bryson et al., 1992). Investigation into the frequency of sexual recombination in the field is currently underway. Preliminary studies on recently collected isolates of *C. truncatum* from Saskatchewan lentil fields show very few polymorphisms in AFLP and SSR bands (Menat, personal communication).

Two distinct races of *C. truncatum* have been identified by Buchwaldt et al. (2004). Initially over 1700 lentil accessions were screened for anthracnose resistance in the field. Under controlled conditions, resistant and moderately resistant accessions were re-tested. Seven accessions were then used as a host differential set to characterize 50 isolates of *C. truncatum* from Manitoba and Saskatchewan. Isolates designated as race Ct1 were avirulent on all seven differentials whereas isolates designated race Ct0 were virulent on all differentials. Pathogenicity testing of the 50 isolates showed race Ct0 occurred more frequently than Ct1 in seed samples, but races were found in equal proportions in the field.

Current investigations by the Pulse Crop Pathology Group at the CDC into field populations of *C. truncatum* with respect to race distribution are underway. These will illuminate the variability of *C. truncatum* populations in Saskatchewan and further understanding of the interaction between *C. truncatum* and *L. culinaris* (Banniza, personal communication). A population of isolates segregating for the races is being pathogenically characterized on a differential set of lentil lines. Preliminary results suggest major gene control of virulence since a bimodal distribution of the isolates on differential line 'CDC Robin' was observed. It is hoped race-specific avirulence genes will eventually be mapped to the *C. truncatum* genome which will assist in monitoring *C. truncatum* populations for changing virulence. Furthermore, *C. truncatum* genes expressed during the infection process are being isolated to identify genes associated with fungal virulence using ESTs of complementary DNA libraries of infected tissue on differential lines of lentil.

Conidia of *C. truncatum* germinate two or three to six hours post-inoculation (hpi) generating germ tubes and appressoria form six to 12 hpi. Direct penetration of

leaf and stem cuticles occurs by infection pegs which result in infection vesicles inside the epidermal cells under the penetration site. Twenty-four hpi, infection hyphae occupy epidermal cells (Chongo et al., 2002; Wang, 2009). Intracellular primary hyphae in the epidermal cells expand in size from 30 hpi then differentiate into different types of primary hyphae at 48 hpi. At 60 hpi, thinner secondary hyphae develop inside local epidermal cells and have spread through the cell walls of adjacent cells 72 hpi (Wang, 2009). Visual appearance of infection of plant tissue is characterized by a symptomless, biotrophic phase for 12 to 72 hours followed by appearance of light greenish, water-soaked lesions that later become necrotic (Chongo et al., 2002). This two-phase infection process is characteristic of many *Colletotrichum* species and is what classifies them as hemibiotrophic (Luttrell, 1974). Appearance of lesions coincides with the necrotrophic phase infection in *Colletotrichum* species (Bailey et al., 1992). Visibility of lesions in susceptible lines occurs two to eight days earlier than on partially resistant lines (Chongo et al., 2002).

Microscopy studies of both races of *C. truncatum* inoculated on differential lentil lines suggest differences in the ability to infect may be due to conidial germination, formation appressoria and development of primary hyphae (Wang, 2009). Resistance to race Ct1 showed delayed growth and spread of hyphae in the plant tissue (Chongo et al., 2002; Wang, 2009) most likely due to the formation of aggregates from primary hyphae in resistant lines (Chongo et al., 2002). Furthermore, accumulation of phenolic compounds was quicker in resistant lines (Chongo et al., 2002). Incubation period and latent period of *C. truncatum* on lines with resistance, such as accession PI320937 and the cultivar 'Indianhead', is longer than on susceptible cultivars, such as 'Eston' (Chongo and Bernier, 1999 and 2000a). Furthermore, lesion number and size, degree of sporulation, disease severity and disease progress is greater for susceptible versus resistant varieties (Chongo and Bernier, 1999 and 2000b).

It is unlikely that seed-borne spread of *C. truncatum* is a major cause of inter-field anthracnose infection as low levels of seed infection are detected even when crops are severely affected (Buchwaldt et al., 1996). For example, in the growing seasons of 2004 and 2005, only 21% and 14% of province-wide seed samples had low

levels of infection, respectively, despite conditions conducive to anthracnose development (Morrall et al., 2005 and 2006). Ascochyta infected 52% and 48% of seed samples in these growing seasons. Wind-spread of anthracnose-infected straw dust generated at harvest, debris and soil dispersion have been identified as sources of inter-field inoculum of *C. truncatum* on the Canadian prairies. Lentil-straw dust was shown to be dispersed more than 240 m from the combine and infected dust was shown to be an effective inoculant. Anthracnose-infected lentil debris on or in the soil is a source of within-field inoculum. Buried, the pathogen can survive up to 44 months as microsclerotia, however only 12 months when on the soil surface (Buchwaldt et al., 1996). Although control of anthracnose can be achieved with foliar fungicides, such as chlorothalonil, and a four year crop rotations with the use of disease-free seed (Buchwaldt et al., 1996; Chongo et al., 1999), use of resistant cultivars is considered to be the best way to manage diseases as it is economical with least environmental impact.

2.4 Resistance to *Colletotrichum*

Colletotrichum truncatum causes anthracnose on many other legume species such as soybean, pigeon pea, lima bean and peanut. In soybean, anthracnose caused by *C. truncatum* is also a major disease which causes infection at all stages of growth especially during flowering to pod fill causing reduction in seed quality, yield and plant death (Lenné, 1992). *Colletotrichum* species also cause anthracnose on various other crops such as maize and sorghum (Ferreira and Warren, 1982; Jamil and Nicholson, 1987; Ngugi et al., 2000; Nicholson et al., 1985), bean, coffee, cashew, cotton, pepper, rubber, sugarcane, tobacco and in tropical fruits such as avocado, banana, citrus, mango and papaya (Waller, 1992).

2.4.1 Lentil

Resistance to race Ct1 has been documented in 16 lines of *L. culinaris* (Buchwaldt et al., 2004) and resistance from 'Indianhead' has been effectively transferred into cultivars such as 'CDC Robin' (Vandenberg et al., 2002) and 'CDC Redberry' (Vandenberg et al., 2006). 'CDC Redberry' is a red cotyledon, small

seeded type and 'CDC Robin' is an extra small red lentil (Table 2.1). It was found that resistance to race Ct1 is conferred by one major gene in accession PI320937 (Buchwaldt et al., 2001; Tullu et al., 2003) and PI345629 and a single recessive gene controls resistance in 'Indianhead' (Buchwaldt et al., 2001). Tullu et al. (2003) named the single dominant resistance gene in PI320937 *LCt-2*, and found that several minor genes also contributed to resistance. RAPD markers $OPE06_{1250}$ and $UBC\text{-}704_{700}$ were found to be linked to *LCt-2* at a distance of 6.4 cM and 10.5 cM. Additionally, three AFLP flanking markers ($EMCTTACA_{350}$, $EMCTTAGG_{375}$ in coupling and $EMCTAAAG_{175}$ in repulsion) for the major gene have been placed on the lentil linkage map (Tullu et al., 2003). $OPE06_{1250}$ can be used in marker assisted selection for resistance to race Ct1 in segregating populations where PI320937 or 'Indianhead' has been used as a source of resistance (Tar'an et al., 2003 and Tullu et al., 2003). Furthermore, markers linked to two ascochyta resistance genes were developed for detecting breeding lines pyramided with resistant genes for both diseases using marker assisted selection (Chowdhury et al., 2001; Ford et al., 1999; Tar'an et al., 2003).

The genetics of resistance to race Ct0 of *C. truncatum* have not been studied extensively and resistance to race Ct0 within the *L. culinaris* primary gene pool is rare. To date, there have been no cultivars or land races of *L. culinaris* identified with resistance to Ct0 with the exception of VIR421 and lines from the family of breeding lines 3155S (Table 2.1). Plant Gene Resources of Canada screened more than 1700 accessions from the Vavilov Institute and identified VIR421 with partial resistance (Buchwaldt, personal communication) which was collected in Afghanistan by Barulina (1930). Breeding lines from 3155S are $F_{2:3}$ family selections from the cross Yerli Kirmizi / 'CDC Redberry' // 'CDC Redberry' that were identified as having high levels of resistance in the CDC anthracnose disease nursery in 2005. The 3155S lines were developed using a form of cyclical recurrent selection utilized at the CDC to enhance germplasm for minor resistance genes to anthracnose (Muehlbauer et al., 2009). Yerli Kirmizi (translated means 'local red'), is a landrace grown extensively in Turkey. Inoculations with *C. truncatum* performed at the onset of this projected showed Yerli Kirmizi was highly susceptible, thus is thought it's contributions to resistance observed in 3155S families was very minimal and limited to minor genes.

Table 2.1. Lines of *Lens culinaris* and *Lens ervoides* differing in resistance to race Ct1 and race Ct0 of *Colletotrichum truncatum* utilized in various studies comparing resistance and elucidating genetic control.

Line	Race Ct1 Resistance	Race Ct0 Resistance	Origin	Pedigree	Cotyledon Colour	Reference
Pardina	Highly Susceptible	Highly Susceptible	Spanish landrace		Red	
Yerli Kirmizi	Highly Susceptible	Highly Susceptible	Turkish landrace		Red	
Eston	Susceptible	Susceptible	Canadian cultivar	Selection from PI79307	Yellow	Slinkard (1981)
Indianhead	Resistant	Susceptible	Canadian cultivar		Red	
CDC Robin	Resistant	Susceptible	Canadian cultivar	CDC Matador // Eston / ESOR-3-6-1	Red	Vandenberg et al. (2002)
CDC Redberry	Moderately Resistant	Moderately Susceptible	Canadian cultivar	1049 F_3 / 819-5R	Red	Vandenberg et al. (2002)
VIR421	Moderately Resistant	Moderately Resistant	Afghani accession		Red	Buchwaldt (2004)
3155S(-1, -5, -6 and -8)	Moderately Resistant	Moderately Susceptible	CDC Breeding Line	Redberry // Redberry / Yerli Kirmizi	Red	
L-01-827A	Resistant	Resistant	*L. ervoides* accession		Red	Tullu et al. (2006)
LR59-81	Resistant	Resistant	*L. ervoides* x *L. culinaris* interspecific RIL	Eston / L-01-827A	Red and Yellow	Fiala et al. (2009)

Buchwaldt et al. (2004) used a mixed qualitative and quantitative trait scale to describe the level of disease infection by *C. truncatum* in lentil: i) Highly resistant plants had no symptoms; ii) Resistant plants had only superficial lesions; iii) Moderately resistant plants had lesions at the stem base; iv) Moderately susceptible plants had a mixture of deep and superficial lesions on the top half of the stem; v) Susceptible plants were described as having deep lesions on the top half of the stem and partial wilting; and vi) Highly susceptible plants had deep and coalesced lesions and wilting. Partial resistance confers resistance to the less virulent race Ct1 or results in a moderately resistant to moderately susceptible reaction as described by Buchwaldt et al. (2004) when inoculated with race Ct0.

Wild species in the secondary gene pool of cultivated lentil have shown resistance to race Ct0 and race Ct1 in greenhouse and field experiments (Tullu et al., 2006). These include lines of *L. ervoides*, *L. lamottei* and *L. nigricans* with *L. ervoides* having the greatest frequency of resistance. Crosses of cultivated lentil with *L. lamottei* and *L. ervoides* are possible through embryo rescue (Cohen et al., 1984; Fratini and Ruiz, 2006; Ladizinsky et al., 1985), and it is hoped that by crossing *L. nigricans* with *L. ervoides*, *L. nigricans* resistance genes can be brought into cultivated lentil background.

After identifying resistance to anthracnose in the *L. ervoides* gene pool (Tullu et al., 2006), the resistant plant L-01-827A (from accession PI72847) was successfully crossed with *L. culinaris* cultivar 'Eston' and an interspecific population called LR59 was developed (Fiala et al., 2009). 'Eston' is a small seeded, yellow cotyledon, green seed coat, early maturing line released in Canada in 1980 (Slinkard, 1981). Ovule rescue was performed on a cross between the two parental lines to obtain a rooted F_1 hybrid plant which produced an F_2 population of 150 from which recombinant inbred lines (RILs) were developed using single seed decent. Due to varying fertility, the population was reduced to 85 stable lines by $F_{7:8}$. L-01-827A was a single, red-cotyledoned plant found to be resistant when 150 susceptible accessions of *L. orientalis* were inoculated with race Ct0. Morphologically, the plant resembled the wild species *L. ervoides*, thus it was re-classified to that species as it was thought the accession may have been a mixtures of *Lens* species (Fiala et al., 2009). Since then,

L-01-827A has been successfully crossed with a pure line from an *L. ervoides* accession (PI78215) confirming the reclassification to *L. ervoides*.

Investigation of the resulting interspecific population with *L. culinaris* line 'Eston' (susceptible to both Ct0 and Ct1) suggested resistance derived from *L. ervoides* accession L-01-827A to each *C. truncatum* race was controlled by two recessive genes, however it was suspected these results were skewed due to segregation distortion (Fiala et al., 2009). However, two RILs from the L-01-827A x 'Eston' (LR59) population have been backcrossed to several lentil cultivars at the CDC and the resistance gene(s) appear to be behaving as a single dominant gene (Vandenberg, personal communication). Thus further investigation of genetic control of *L. ervoides*-derived resistance in a *L. culinaris* genetic background is required to fully understand the genetics controlling the trait.

An additional interspecific population was developed with the *L. ervoides* accession PI72815 x 'Eston' (LR26) and transfer of resistance genes is being confirmed (Tullu, personal communication). Preliminary results of *C. truncatum* resistance screening of the LR26 recombinant inbred lines (RILs) suggest a similar mode of inheritance to LR59 (Tullu, personal communication). Also, a resistant accession of *Lens lamottei* has been crossed with *C. truncatum* (line 971-16) and the resulting population is another source of unique resistance genes (Fiala, 2006). Susceptible *L. culinaris* line 971-16 and race Ct1 resistant / race Ct0 moderately resistant *L. lamottei* line IG110811 (ILWL 430) were crossed and resistance to race Ct0 was identified in the interspecific progeny (Fiala, 2006). Pollen studies on a line from this cross indicated that pollen was viable and pollen tube growth occurred at a rate similar to parental line when the line was self pollinated. The population has also been cytogenetically characterized and abnormal chromosomal rearrangements during meiosis were observed.

2.4.2 Bean

Colletotrichum lindemuthianum on *Phaseolus vulgaris* is one of the best defined and longest studied *Colletotrichum* species and this pathosystem has served a model for pathogenic, cytological, physiological and molecular studies on host-

pathogen specificity (Bryson et al., 1992; Kelly and Vallejo, 2004). Resistance to infection by *C. lindemuthianum* is characterized by early host cell death at the point of pathogen penetration, inhibition of pathogen growth in the penetrated tissue and limitation of lesion expansion (Bailey and Rowell, 1980). Specific pathogen races trigger resistance to *C. lindemuthianum* in *P. vulgaris* (de Meaux and Neema, 2003) and an international standard differential set of 12 differential host lines from different gene pools containing different arrays of resistance genes exist to classify *C. lindemuthianum* races with a binary code system (Kelly and Vallejo, 2004). Initially, a gene-for-gene interaction with the pathogen and host was assumed, but allelism studies showed avirulence to different sources of resistance is caused by unlinked genes (Bryson et al., 1992).

Use of host resistance is recognized as the most effective method of controlling anthracnose in common bean. Kelly and Vallejo (2004) reviewed the nine major independent genes providing resistance to different races of *C. lindemuthianum* and since then, one more locus conferring resistance to *C. lindemuthianum* has been identified (Gonçalves-Vidigal et al., 2008). Alternate alleles providing different levels of resistance have been found for four of the resistance loci. Resistance is generally dominant, however one locus has a multi-allelic series where different alleles confer different levels of dominance. Conversely, an independent loci has a recessive resistance gene. Many of the genes have been mapped to an integrated bean linkage map using a range of marker techniques. They tend to be linked to resistance genes for other pathogens in the *Phaseolus* genome (Kelly and Vallejo, 2004). As well, ten QTLs have been identified throughout the bean genome. These appear to be involved in partial resistance, some of which seem to be race non-specific (Geffroy et al., 2000). Despite the wide range of sources of resistance to anthracnose in bean, breeders struggle with decisions on how to effectively deploy resistance gene(s) (Kelly and Vallejo, 2004).

Two *C. lindemuthianum* resistance loci in *P. vulgaris* have been the focus of map-based cloning efforts, the *Co-2* locus which was originally considered to be race non-specific and the *Co-4* locus which has an allele, $Co\text{-}4^2$, that provides resistance to a highly virulent race of *C. lindemuthianum* (Kelly and Vallejo, 2004). Candidate

resistance gene analogues with leucine rich repeats (LRR) and kinase protein domains have been reported for *Co-2* (Geffroy et al., 1998; Creusot et al., 1999) and a gene coding for a serine-threonine kinase domain, similar to the *Pto* gene in tomato, but with a highly hydrophobic membrane-spanning region was found using a tightly linked marker to *Co-4*^{2} (Melotto and Kelly, 2001). A complex, ancestral resistance gene cluster has been identified at the end of a specific linkage group (Geffroy et al., 1999) with both Middle American and Andean resistance genes and QTLs mapped to the region (Geffroy et al., 2000). Four resistance gene candidates that code for nucleotide binding site-LRR proteins have been characterized (Ferrier-Cana et al., 2003). This cluster is being used to investigate the evolution of the two gene pools of *P. vulgaris* and resistance gene cluster evolution (Ferrier-Cana et al., 2003; Geffroy et al., 2009).

Most *P. vulgaris* resistance genes for *C. lindemuthianum* are of Middle American in origin with only two from the Andean gene pool (Gonçalves-Vidigal et al., 2008; Kelly and Vallejo, 2004). A need for diverse sources of resistance is widely recognized among bean breeders. Wild beans from the primary gene pool of *P. vulgaris* are not considered to be a source of stable resistance, but potential for resistance lies within the secondary gene pool of *P. vulgaris*. Screening of 93 *Phaseolus coccineus* accessions showed 98% were highly resistant and out of 69 accessions of *Phaseolus polyanthus*, 93% were immune even to the most highly virulent race of *C. lindemuthianum*. Abundant resistance was also found in the interspecific lines with resistance being especially high when *P. polyanthus* was one of the parents. Genes found within the secondary gene pool are transmissible to common bean (Mahuka et al., 2002). Resistance to ascochyta blight (Schmit et al., 1992), angular leaf spot (Mahuku et al., 2003) and white mold (Hunter et al., 1982) have also been identified in the secondary gene pool and in some instances resistance genes have been introgressed into *P. vulgaris* (Singh et al., 2009).

2.5 Plant Age and Disease Resistance

Stage, phase or age-specific expression of disease resistance has been shown in various crop species and is considered a limiting factor in yield improvement (Whalen,

2005). Resistance gene products have an evolutionary relationship to developmental gene products and it is thought that both may have evolved from a common ancestral gene which performed different functions (Whalen, 2005). However, there is overall very little understanding of the underlying mechanisms of the relationship between plant development and disease resistance (Chintamanani et al., 2008; Develey-Rivière and Galiana, 2007).

Developmental resistance has been defined as changes in resistance to a pathogen that are correlated with the developmental stage of the host or of plant organs with changes in resistance that can be gradual or abrupt (Whalen, 2005). Increase in acquisition of resistance has been coined with many names including juvenile (de Souza and Café-Filho, 2003), ontogenic, developmental, seedling, mature-seedling, adult-seedling, age-related, flowering-induced and senescence-induced resistance (Develey-Rivière and Galiana, 2007; Mew, 1987). Most common is the acquisition of resistance with age (Develey-Rivière and Galiana, 2007). The mechanisms that have been shown to operate developmental control of resistance are diverse across pathosystems and are not associated with a particular class of resistance gene (Whalen, 2005). It is expected that expression of resistance genes may not be directly under developmental control, but competency factors could be. For example, developmental control could exist for a resistance gene product that is involved in recognition of the pathogen's effectors or increased stability of recognition could occur with development (Whalen, 2005).

One of the best understood pathosystems exhibiting developmental resistance is that of rice and *Xanthomonas oryzae* pv. *orzae*. Extensive studies on the effect of plant age on resistance to *X. oryzae pv. orzae* in various lines have shown that genes that confer resistance in the seedling stage generally continue to confer resistance in adult plants, however various adult resistance genes do not provide resistance at the seedling stage (Mew, 1987). It is generally thought that seedling resistance to *X. oryzae pv. orzae* is not as stable as that of adult resistance, however this seems to depend on the combination of cultivar and isolate being examined (Mew, 1987). The *X. oryzae pv. orzae* resistance gene *Xa21* does not provide resistance to the pathogen in early stages of development (Mazzola et al., 1994) even though the expression of

the resistance gene in susceptible younger leaves was found to be the same as resistant older leaves (Century et al., 1999). It was suggested this was due to post-transcriptional regulation of the resistance gene product (Century et al., 1999). So far, the signalling pathway initiated by *Xa21* is not well understood but it is hoped that insight into the functional overlap, crosstalk and evolutionary relationships between the two pathways will be gained once the signalling pathway is resolved (Whalen, 2005).

Similarly in maize, *Hm2* confers resistance to *Cochliobolus carbonum* race 1 only at maturity. Both resistance of the plant and expression of *Hm2* were higher in homozygous versus heterozygous plants, but transcripts of the gene did not vary based on stages of development. *Hm2* is a duplicate of *Hm1* which exhibits completely dominant resistance at all stages of plant development. *Hm2* encodes a shorter protein than the *Hm1* resistance gene suggesting an explanation for the disease resistance phenotype observed (Chintamanani et al., 2008).

However, when susceptible lentil line 'Eston' and *C. truncatum* race Ct1 resistant lines 'Indianhead' and 458-57 were inoculated with *C. truncatum* race Ct1, Chongo and Bernier (2000a) found that anthracnose resistance was greatest at the seedling stage and plants were mostly susceptible at the 8 to 10 node stage four weeks after planting. Susceptibility decreased through the flowering and early podding stages, but not to the level of resistance observed in the seedlings. This was consistent across several components of partial resistance as incubation period, latent period, the number of lesions per stem and disease severity were all assessed. The difference between susceptible 'Eston' and race Ct1 resistant lines was greatest at the eight to ten node stage (Chongo and Bernier, 2000a).

Seedling or juvenile resistance that decreases as the plant ages has also been reported from other pathosystems, however is not as common or as well studied as developmental resistance. For example, resistance decreases with plant age for spot blotch in wheat (Vergnes et al., 2006), powdery mildew resistance in pepper (de Souza and Café-Filho, 2003) and anthracnose in sorghum (Ferreira and Warren, 1982; Ngugi et al., 2000) and maize (Jamil and Nicholson, 1987; Nicholson et al., 1985). Postulated reasons for a decline in resistance include physiological changes associated

leaf senescence and source-sink metabolism, increased sensitivity to pathogen phytotoxins (Vergnes et al., 2006), decreasing cyanogenic precursors reducing plant defence mechanisms (Ferreira and Warren, 1982) or high concentrations of inhibitory compounds, such as phenolic compounds, tannins and dienes, which decrease in concentration as the plants age (Agrios,1997). Different genes controlling resistance in seedling or juvenile versus adult or reproductive phases have been found for turnip mosaic virus resistance in Chinese cabbage (Zhang et al., 2008), resistance to *Cochliobolus sativus* (Pathotype 1), causing spot blotch in barley (Bilgic et al., 2006) and for seedling, flag leaf and glume resistance to *Stagonospora nodorum* in wheat (Shankar et al., 2008).

CHAPTER 3

FIELD EVALUATION OF RESISTANCE TO *COLLETOTRICHUM TRUNCATUM* IN *LENS CULINARIS*, *LENS ERVOIDES* AND *LENS ERVOIDES* X *LENS CULINARIS* LINES

3.1 Introduction and Objectives

Abundant resistance to *C. truncatum* race Ct0 was identified by Tullu et al. (2006) in accessions of *Lens ervoides* in field disease nurseries in 2000 and 2002 and was confirmed with inoculations under controlled conditions indoors. Based on those results, a population of interspecific recombinant inbred lines (RILs), LR59, from a cross between a resistant *L. ervoides* plant and a susceptible *L. culinaris* plant was developed. Highly resistant lines to both races of *C. truncatum* were identified when inoculated under controlled conditions, however resistance was not tested under field conditions in that study (Fiala et al., 2009). Field characterization for resistance to *C. truncatum* in LR59 RILs, and select lines of *L. culinaris* and *L. ervoides*, were performed in the current study across two sites and two growing seasons. The objectives were to determine the potential usefulness of the resistance genes from *L. ervoides* accession L-01-827A, to estimate the heritability of the resistance and to compare the level of resistance with other sources of resistance found in *L. ervoides* and *L. culinaris*.

3.2 Materials and Methods

3.1.1 Plant material

Thirty lentil lines were evaluated at two sites in the growing seasons of 2006 and 2007. Fourteen $F_{7:9}$ recombinant inbred lines (RILs) and the parent lines from the interspecific cross 'Eston' / L-01-827A (LR59) with differing levels of resistance to

race Ct0 and race Ct1 (Fiala et al., 2009) were assessed in the field. Also included were four $F_{2:4}$ (2006) or $F_{2:5}$ (2007) families from the cross 3155S (CDC Redberry // CDC Redberry / Yerli Kirmizi) along with the parents 'CDC Redberry' and Yerli Kirmizi. A series of resistant and susceptible *L. culinaris* checks were also included as a means of linking the results to previous research on anthracnose resistance in lentil. The susceptible check 'Pardina' is a Spanish landrace grown in both Spain and in the Palouse area of the north-western USA. It has been used as a universal susceptible check for both ascochyta blight and anthracnose in the CDC lentil breeding program for many years thus was included (Table 2.1). Partially resistant lines 'CDC Robin' (Vandenberg et al., 2002), VIR421 (Buchwaldt and Diederichsen, 2004) and 'Indianhead' were included along with four additional accessions of *L. ervoides* (PI72815, PI7250-3, PI72659 and PI116015) that were selected based on previous reports of anthracnose resistance (Tullu et al., 2006).

3.2.2 Disease nurseries, experimental design and disease inoculation

Sites of the experiments were both in Saskatoon at the North Seed Farm (NSF) and Preston farms of the Department of Plant Sciences, University of Saskatchewan, Saskatoon, Saskatchewan. The NSF site is a lentil disease nursery that has been used by the Crop Development Centre (CDC) lentil breeding program for many years and is presumed to be a mixture of *C. truncatum* races. Lentil breeding lines have been inoculated with the previous year's infested lentil stubble for more than 12 years. The field is cultivated prior to seeding so the soil is also infested with *C. truncatum* . The NSF was seeded May 25th in 2006 and May 17th in 2007. The seeding date of the Preston site was deliberately delayed to help ensure a contrasting environmental effect based on precipitation pattern and was June 2nd both years. The plots were arranged as a randomized complete block design with four replications per site each year. Plots were one row, 75 cm long and had 20 seeds per plot planted approximately 2.5 cm deep with a row cone drill with 30 cm between rows.

Each plot was flanked with rows of both 'Eston' (susceptible to both races) and 'CDC Redberry' (moderately resistant to Ct1 and moderately susceptible to Ct0). Plots were inoculated on July 4th in 2006 and June 29th in 2007 with diseased lentil

straw that had been collected from the NSF anthracnose nursery the previous year. Misting irrigation was used to promote disease development. It was applied only in the evening, overnight and early morning at the NSF. The Preston location had a misting irrigation system that ran regularly over a 24 hour period. Each replication at all sites was surrounded by four rows of barley to prolong canopy wetness throughout the day.

3.2.3 Disease evaluation and analysis

Whole plots were rated once a week throughout the epidemics, starting July 18th and July 25th in 2006 at NSF and Preston sites for 38 and 48 days, respectively and starting July 13th at both sites in 2007 for 22 days. The scale described by Horsfall and Barratt (1945) is based on grades of disease that differ by a factor of two either side of 50% diseased tissue. The theory underlying the grades is that the human eye distinguishes diseased or disease-free tissue according to the logarithm of light intensity. This scale was used to obtain percentage grade values for the amount of plant tissue infected with disease. These were converted to Area Under the Disease Progress Curve (AUDPC; Shaner and Finney, 1977) values and to compare AUDPC values from different sites and years, values were converted to relative AUDPC (rAUDPC) values by dividing the AUDPC values by the duration of the epidemic and multiplying the outcome by 100 as described by Shtienberg et al. (2000). Values were analyzed as a combined experiment in the Mixed procedure in SAS (SAS Institute, Cary, NC) in a model where all factors were considered random (Site, Year, Replication within each Site and Year and all interactions) except for Line which was considered a fixed effect. The disease ratings of the flanking resistant and susceptible check plots were added to the statistical model as resistant and susceptible covariates to adjust for within-block variability. Covariates were found to be highly significant and greatly reduced the contribution of the site years to overall variability suggesting variable disease severity within each of the sites (Appendix 1 to 5). Separate analyses by year and by each site within each year were also performed using the Mixed procedure. Least square means were calculated and standard errors of differences and

p-values for differences between pairs of means of different lines were calculated using the PDIFF option.

For the set of lines that were common to previous indoor rating experiments by Fiala et al. (2009), Pearson correlation coefficients were calculated in SAS for average field disease ratings and those at each site in each year to the previous indoor ratings which were inoculated separately with both races of the pathogen. Relative AUDPC values of the 14 LR59 RILs were also used to calculate the heritability of anthracnose resistance using the method described by Capettini et al. (2003). Plot values were subject to analysis of variance using the general linear model procedure in SAS. Significance of the factors was tested using the test option. Expected mean squares (Table 3.3) were used to calculate appropriate variances which were used to calculate the genetic variance due to lines and the overall phenotypic variance and the ratio between the values.

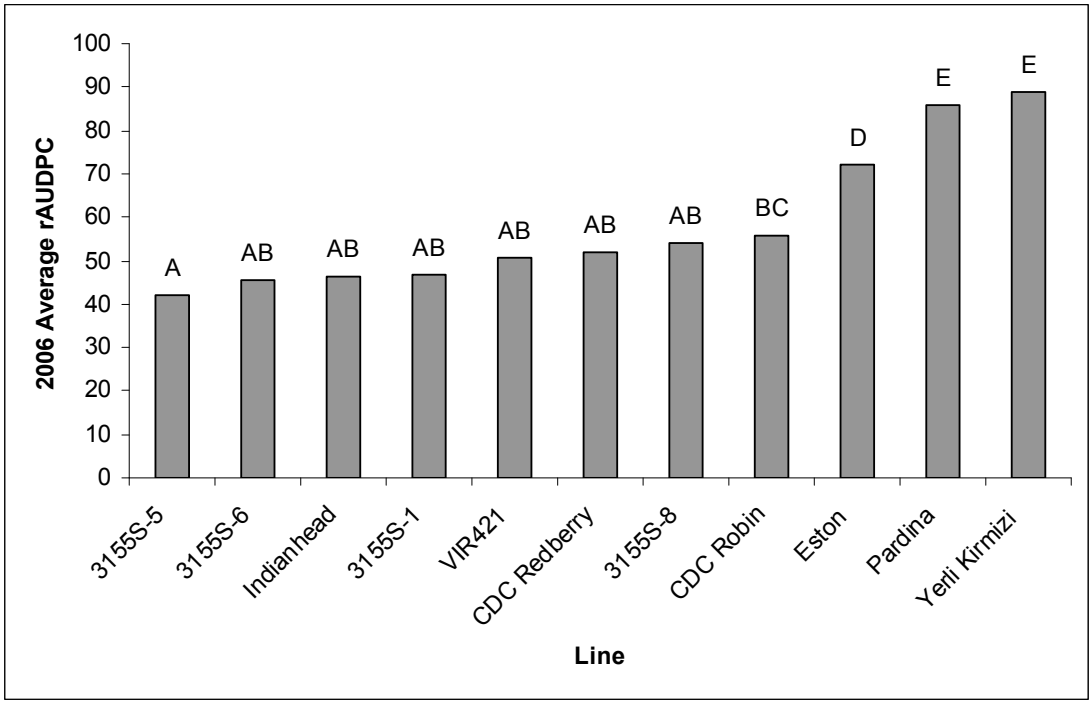
3.3 Results and Discussion

Disease severity was greater in the growing season of 2006 than in 2007 at both sites, most likely due to more in-season rainfall in 2006. In both years, the NSF site, which had a much longer history of anthracnose inoculation activity, had more disease than Preston. The susceptible check 'Eston' had maximum disease at the end of the epidemic at both sites in 2006, where in 2007 only had 87% of the plants with disease at the NSF and 20% at Preston. When all lines tested were considered, highly significant differences were observed among lines in both seasons at both sites (Appendix 1). However, differences within each of the groups of lines (*L. culinaris*, *L. ervoides,* 3155S *L. culinaris* $F_{2:4}$ or $F_{2:5}$ families and interspecific RILs) significant differences between lines were consistently absent in 2007 (Appendix 2 to 5). The commencement of flowering was delayed, on average, around two weeks at the Preston site compared to the NSF site, which may also have contributed to reduced disease levels. Chongo et al. (1999) showed the positive effect of partial resistance to anthracnose on reducing yield loss under high disease pressure. Under high disease pressure, partially resistant cultivars in combination with fungicide treatment showed less yield loss and lower disease than susceptible cultivars with fungicides applied

Chongo et al. (1999). However, under low to moderate anthracnose pressure, as was observed in the 2007 growing season of this study, there was no benefit to partial resistance over susceptible cultivars.

It was expected based on observations in the anthracnose nursery in 2005 that families from cross 3155S would show improved resistance over commercially available partially resistance lines. However, the results of the disease ratings in 2006 suggest similar resistance compared to the resistant recurrent parent 'CDC Redberry' (Figure 3.1). It was evident that the lines in this family did not acquire additional major resistance genes from the exotic parent, Yerli Kirmizi. The family 3155S-5 showed a small improvement ($p < 0.05$) over the race Ct1 resistant line 'CDC Robin' (Figure 3.1) suggesting that variation for resistance from this cross may be useful for cultivar improvement in the lentil breeding program at the CDC. Differences among the four families of the 3155S cross were not observed in either season in this experiment (Appendix 2). It is possible that this small sub-sample (four of approximately 75 $F_{2:3}$ families) failed to capture the variability in the larger population. Furthermore, susceptibility within each of the families would have previously been selected against in the growing season of 2005 when grown in the disease nursery.

Figure 3.1. Relative area under disease progress (rAUDPC) curves for all lines of *Lens culinaris* evaluated in anthracnose nurseries in 2006.



Note: Significant differences ($p < 0.05$) of pair wise contrasts used to group disease reaction differences

When all *L. culinaris* lines were considered, two clear defined groups were observed based on ratings of anthracnose in 2006 (Appendix 3, Table 3.1 and Figure 3.1). Moderate resistance was observed in the group of currently available cultivars classified as being resistant to race Ct1 compared to the susceptible group 'Eston', 'Pardina' and Yerli Kirmizi. The accession VIR421, which was identified by Plant Gene Resources Canada as having some resistance to race Ct0, had field resistance similar to that exhibited by the other *L. culinaris* lines that were rated as race Ct1 resistant and partially resistant to Ct0. When representative lines from each of the groups were compared (Table 3.1 and Figure 3.2), the resistant LR59 RILs had significantly greater resistance than the most resistant *L. culinaris* lines (3155S-5, VIR421 and 'CDC Redberry') suggesting that this source of resistance could be effectively used to reduce the incidence and severity of anthracnose in lentil crops in

Saskatchewan. Furthermore, variation found within the small group of *L. ervoides* lines previously screened for anthracnose resistance (Appendix 4) suggests that even higher levels of resistance are available in this species. The accession PI72815 had significantly less disease than the resistant *L. ervoides* parent L-01-827A (Table 3.1 and Figure 3.2), consistent with average ratings of Tullu et al. (2006) who found PI72815 was one of the most resistance *L. ervoides* accessions. Resistance genes from PI72815 are currently being transferred to *L. culinaris* by use of interspecific RILs from a cross with 'Eston' (Tullu, personal communication).

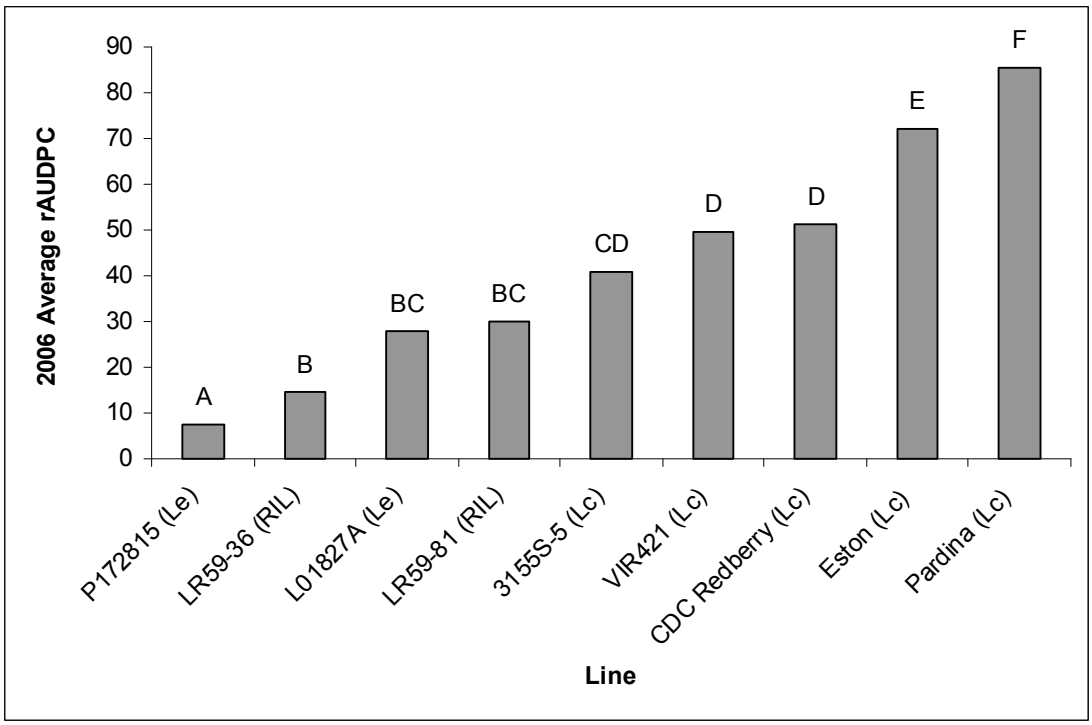
Table 3.1. Relative area under disease progress curves (rAUDPC) for lines of lentil evaluated in anthracnose nurseries at the North Seed Farm (NSF) and Preston sites in the growing seasons of 2006 and 2007.

				2006 rAUDPC		2007 rAUDPC	
	Line	**Species**	**Average rAUDPC**	**NSF**	**Preston**	**NSF**	**Preston**
LR59 Interspecific Recombinant Inbred Lines	LR59-25	*L. ervoides* x *L. culinaris* RIL[†]	23.1	49.2	36.4	5.2	1.1
	LR59-31	*L. ervoides* x *L. culinaris* RIL	40.6	79.4	47.4	31.4	4.2
	LR59-33	*L. ervoides* x *L. culinaris* RIL	29.8	66.4	39.3	8.6	3.4
	LR59-36	*L. ervoides* x *L. culinaris* RIL	8.6	18.6	10.9	1.8	2.5
	LR59-38	*L. ervoides* x *L. culinaris* RIL	9.9	24.9	10.9	1.8	1.7
	LR59-54	*L. ervoides* x *L. culinaris* RIL	17.3	48.3	15.8	3.2	0.9
	LR59-76	*L. ervoides* x *L. culinaris* RIL	19.8	55.6	14.2	5.8	1.7
	LR59-80	*L. ervoides* x *L. culinaris* RIL	21.3	59.9	21.2	4.5	1.1
	LR59-81	*L. ervoides* x *L. culinaris* RIL	15.9	39.7	20.5	2.9	0.7
	LR59-87	*L. ervoides* x *L. culinaris* RIL	10.1	32.5	1.8	2.0	2.0
	LR59-91	*L. ervoides* x *L. culinaris* RIL	31.2	72.2	45.1	9.9	2.3
	LR59-105	*L. ervoides* x *L. culinaris* RIL	32.7	68.6	46.5	11.2	3.6
	LR59-132	*L. ervoides* x *L. culinaris* RIL	35.4	73.6	43.3	22.9	3.2
	LR59-133	*L. ervoides* x *L. culinaris* RIL	34.9	73.7	43.3	18.5	2.0
Commercial Lentil Cultivars	Yerli Kirmizi	*L. culinaris*	65.8	96.3	79.9	69.5	17.8
	Eston	*L. culinaris*	48.1	85.2	58.8	39.8	9.0
	Indianhead	*L. culinaris*	25.8	63.6	28.9	9.3	4.0
	Pardina	*L. culinaris*	62.0	95.7	74.9	63.2	16.9
	CDC Redberry	*L. culinaris*	28.5	71.5	31.1	10.0	3.7
	CDC Robin	*L. culinaris*	33.4	75.4	35.0	17.9	6.3
	VIR421	*L. culinaris*	28.2	67.8	31.2	9.5	3.5
Lentil Breeding Lines	3155S-1	*L. culinaris* F_4 or F_5[‡]	25.9	64.4	28.0	10.4	4.0
	3155S-5	*L. culinaris* F_4 or F_5	24.2	56.2	25.7	8.0	4.8
	3155S-6	*L. culinaris* F_4 or F_5	26.7	68.6	21.9	10.5	6.2
	3155S-8	*L. culinaris* F_4 or F_5	29.8	71.5	36.4	9.6	4.3
Lens ervoides accessions	PI72570-3	*L. ervoides*	12.3	42.9	2.9	3.0	1.6
	PI116015	*L. ervoides*	68.1	96.3	81. 7	70.3	22.7
	PI72659	*L. ervoides*	19.7	57.7	14.7	2.7	2.1
	L-01-827A	*L. ervoides*	15.4	39.5	16.8	3.0	1.3
	PI72815	*L. ervoides*	5.6	10.5	4.9	3.0	2.2

[†]*Lens ervoides* accession L-01-827A x *Lens culinaris* accession 'Eston' Recombinant Inbred Lines (RILs)

[‡]Yerli Kirmizi / 'CDC Redberry' // 'CDC Redberry' $F_{2:4}$ (in 2006) or $F_{2:5}$ (in 2007)

Figure 3.2. Relative area under disease progress curves (rAUDPC) for representative lines of *Lens ervoides* and *Lens culinaris* and interspecific recombinant inbred lines (RIL) evaluated in anthracnose nurseries in 2006.



Note: Significant differences ($p < 0.05$) of pair wise contrasts used to group disease reaction differences

Differences in anthracnose ratings between lines from the LR59 population were most evident under higher disease pressure in 2006. These were not as evident in the 2007 or combined data (Appendix 5). The highly resistant lines from the LR59 population (LR59-36, -38, -87, -81, -54, -76 and -80) showed similar resistance to the *L. ervoides* parent L-01-827A. The susceptible RILs (LR59-31, -132, -133 and -91) had similar ratings compared to the susceptible parent 'Eston' (Table 3.1). Transgressive segregants for resistance or susceptibility were not observed in the field for this population as were observed by Fiala et al. (2009) when inoculated under controlled conditions. High positive correlations existed between relative AUDPCs at each site in each year and the average ratings from indoor screening performed by Fiala et al. (2009; Table 3.2). Correlations were slightly higher in 2006 under greater

disease pressure, however all values were highly significant. This suggests that field evaluation of resistance accurately reflects resistance to both races in this population even under lower disease pressure. These results support use of the anthracnose nursery for selection of resistance derived from L-01-827A. Similarly, Chongo and Bernier (1999) found significant correlations between field inoculations and disease screening under controlled conditions for various components of partial resistance. Resistance derived from L-01-827A to both races of *C. truncatum* is controlled by the same gene(s), or by genes that are tightly linked (as described in Chapter 6). Thus it is not surprising similar results were observed from field inoculations with a population of mixed races, and indoor inoculations with individual races.

Table 3.2. Correlations of relative area under disease progress curve values from field experiments with indoor testing done by Fiala et al. (2009) of 14 interspecific RIL lines inoculated with race Ct1 and race Ct0 of *Colletotrichum truncatum*.

	Race Ct1		Race Ct0	
	Correlation †	***p*-Value**	**Correlation** †	***p*-Value**
Average	0.85	<0.01	0.81	<0.01
North Seed Farm 2006	0.76	<0.01	0.75	<0.01
Preston 2006	0.90	<0.01	0.84	<0.01
North Seed Farm 2007	0.70	<0.01	0.66	<0.01
Preston 2007	0.69	<0.01	0.71	<0.01

† Pearson correlation coefficient

Interactions of the line by year and line by year by site were highly significant and accounted for approximately 10% of the variability observed (Table 3.3). However, site by line interaction was absent. Small differences between recombinant inbred lines ($p < 0.10$) were observed over all sites and years (Table 3.3 and Appendix 5). The estimate of heritability was calculated to be 0.56. Selection for resistance derived from L-01-827A based on single plants at the F_2 and F_3 generations is now regularly employed at the NSF as part of the CDC lentil breeding program. These results confirm that single plant selections may be effective as the heritability is

moderate. This should be confirmed, however, at a later generation when replicated trials are possible. Based on field observations, it was expected that heritability would be higher than calculated. The moderate heritability estimate may have been a result of lower disease pressure observed in 2007 or lower disease levels observed at the Preston site both years, and thus it may be a true reflection of the nature of detecting inheritance of this trait when observed over field seasons with varying conditions. Chongo and Bernier (1999) suggested that AUDPC values from the field were useful to compare and select resistant lines, but when evaluating components of partial resistance under field conditions, more than two years of testing may be required. Heritability values for disease resistance in interspecific populations in other crop species range from very low in tomato to *Phytophthora infestans* derived from *Solanum habrochaites* (Abreu et al., 2008) to high in soybean to *Phytophthora sojae* derived from *Glycine soja* (Tucker et al., 2010). Moderate narrow-sense heritability for resistance to Pierce's disease, caused by *Xylella fastidiosa*, was also observed in an interspecific *Vitis* population (Krivanek et al., 2005).

Table 3.3. Combined analysis of variance, estimates of variance components and heritability for interspecific recombinant inbred lines tested for anthracnose resistance at two sites in the growing seasons of 2006 and 2007.

Source	df	Sum of Squares †	Mean Square ‡		Expected df ¶	Expected Mean Square
Site (S)	1	98125024	98125024		*s*-1	
Year (Y)	1	832633372	832633372**		*y*-1	
S*Y	1	21336929	21336929**		(*s*-1)(*y*-1)	
S*Y(Rep)	12	15911060	1325922**		*sy*(*r*-1)	
Line (L)	13	291296809	22407447*	M5 §	*l*-1	$\sigma^2_e + r\sigma^2_{lsy} + ry\sigma^2_{ls} + rs\sigma^2_{ly} + rsy\sigma^2_l$
Y*L	13	131089161	10083782***	M4	(*l*-1)(*y*-1)	$\sigma^2_e + r\sigma^2_{lsy} + rs\sigma^2_{ly}$
S*L	13	23241335	1787795	M3	(*l*-1)(*s*-1)	$\sigma^2_e + r\sigma^2_{lsy} + ry\sigma^2_{ls}$
S*Y*L	13	26232842	2017911***	M2	(*l*-1)(*s*-1)(*y*-1)	$\sigma^2_e + r\sigma^2_{lsy}$
Residual	154	111805517	726010	M1	*sy*(*l*-1)(*r*-1)	σ^2_e
σ^2_g § = 784611						
H^2 # = 0.56						

† Type III Sum of Squares;

‡ Significant source of variation, * P<0.10, ** $P < 0.05$, *** $P < 0.01$.

§ σ^2_e = M1, σ^2_{lsy} = (M2-M1)/r, σ^2_{ls} = (M3-M2)/ry, σ^2_{ly} = (M4-M2)/rs, $\sigma^2_g = \sigma^2_l$ = (M5-M3+M2-M4)/rsy,

$\sigma^2_p = \sigma^2_g + \sigma^2_{lsy}/sy + \sigma^2_{ls}/s + \sigma^2_{ly}/y + \sigma^2_e/rsy$

¶ r = number of repetitions, y = number of years, s = number of sites, l = number of lines

$H^2 = \sigma^2_g/\sigma^2_p$

3.4 Conclusions

It was found that *L. ervoides*-derived resistance to anthracnose appears to show the highest level of field resistance compared to resistance found within the *L. culinaris* genepool, especially under higher disease pressure as was observed at the NSF site. Usefulness of the introgressed resistance genes will be especially evident in seasons conducive to anthracnose development, as was observed in the growing season of 2006. Based on the results of this study, it is suggested that selection for resistant plants from F_2 disease nurseries be complemented with indoor testing at another generation as field seasons with non-conducive conditions for anthracnose, such as 2007 in this study, may not fully amplify differences in resistance thus ensure selection for highly resistance plants or lines.

3.5 Prologue to Chapter 4

The results from the field study warranted continued investment into introgression of resistance genes from *L. ervoides* accession L-01-827A into cultivated lentil. Due to the suspected distortion associated with the interspecific LR59 population, it is imperative to use an early generation population for phenotypically characterizing genetic control of resistance. However, single plant phenotyping of resistance in this pathosystem can be unreliable and inconsistent. Clonal propagation of single plants has been used for various pulse crops to increase populations sizes. It was proposed this technique could be used to generate many plants of a single F_2 genotype so replicated disease resistance data could be obtained, thus the study described in Chapter 4 was initiated.

CHAPTER 4

EVALUATION OF A CLONAL PROPAGATION PROTOCOL TO OBTAIN REPLICATED DISEASE DATA ON INFECTION BY *COLLETOTRICHUM TRUNCATUM* IN *LENS CULINARIS*

4.1 Introduction and Objectives

The following study was initiated at the onset of this project in order to establish a protocol for use in subsequent studies. The work was recently published (Vail and Vandenberg, 2010) as a methodology prelude to the work presented in Chapter 6.

The results of the field study showed that resistance in some *L. ervoides* x *L. culinaris* recombinant inbred lines (RILs) was much greater than that found within the *L. culinaris* background. Due to the suspected distorted nature of the raw interspecific population as reported by Fiala et al. (2009), the genetic control of resistance needs to be examined in a *L. culinaris* background. Selection of improved resistance is preferred in early generations of segregating populations in the CDC lentil breeding program. Early generation populations are sometimes more appropriate for the study of disease resistance genetics, especially when examining interspecific-derived genes. However, consistency in phenotyping disease resistance to *Colletotrichum truncatum* in lentil on the basis of rating reactions of a single plant is difficult, mainly because of false incompatible reactions and varying levels of susceptibility. This is due to the effect of microenvironment and other experimental variables, even when testing is conducted under controlled conditions in a greenhouse or growth chamber. Detached leaf assays are commonly employed for phenotyping disease resistance in early generations of segregating populations in genetic studies. For example, detached leaves have been used to assess anthracnose resistance in common bean (*Phaseolus*

vulgaris L.) (Tu, 1986) and chocolate spot in faba bean (*Vicia faba* L.) (Bouhassan et al., 2004).

Inoculating detached leaves of various *Lens culinaris* lines with race Ct0 of *C. truncatum* can differentiate resistance from susceptibility; however, this method cannot clearly discriminate partially resistant lines from highly resistant lines (unpublished data). Furthermore, cytological differences between common lines inoculated with both races of *C. truncatum* have been noted using intact leaves compared to detached inoculated leaves (Wang, 2009). Significant differences in defence responses between detached and attached leaf assays were found in *Arabidopsis thaliana* inoculated with *Colletotrichum higginsianum* (Liu et al., 2007). Both salicylic acid– and ethylene-dependent pathways were required for resistance to *C. higginsianum* in *A. thaliana* in intact plants; detaching the leaves interfered with the ethylene pathway, resulting in false compatible reactions (Liu et al., 2007). Thus, obtaining disease resistance values on replicated whole plants is essential, especially for *Colletotrichum* pathosystems.

Recombinant inbred lines are often used to obtain replicated phenotypic data for disease resistance in segregating populations for genetic studies. However, the development of RILs is demanding of both time and resources. Micropropagation has been useful in increasing production of F_2 seed in intra- and interspecific *Lens* population development (Fratini and Ruiz, 2008) and is often used in lentil breeding and genetic research programs for boosting population sizes by increasing the number of plants of a single genotype. As this method is relatively simple, it could be used to generate plants to obtain replicated disease data for a single F_2 genotype. This method would facilitate collection of both replicated F_2 disease reaction data and production of F_3 seed from a single plant, which would prove to be very useful in genetic studies. A similar method was used for testing resistance to *Ascochyta rabiei* in *Cicer arietinum* (Tar'an et al., 2007).

The objective of this study was to evaluate use of clonal propagation to generate replicated data for single plants for scoring resistance to *C. truncatum* . The method was first evaluated on a range of lines with varying levels of resistance, then on an F_2 population segregating for resistance to the pathogen. Developmental and

plant architectural differences between plants grown from seeds vs. cuttings were also assessed on the F_2 population to determine what differences exist and to see if they are associated with disease resistance classification.

4.2 Materials and Methods

4.2.1 Cutting protocol

Cutting-derived plants were developed by first growing mother plants from single lentil seeds in an eight hour photoperiod for six weeks (21°C Day : 15°C Night) in a GR48 growth room (Conviron, Controlled Environments Limited, Winnipeg, MB). The purpose of the short photoperiod was to prolong the vegetative phase of the mother plants, allowing plants to grow new branches and more vegetative tissue so that subsequent cuttings could be taken. Cuttings were then taken from the first node below the youngest fully expanded leaf and treated with 100 mg of STIM-ROOT No.1 (Indole-3-butyric acid 0.1%) (Nu-Gro IP Inc., ON) before placing in a Jiffy-7 peat pellet (Jiffy Products, NB). Ten days after cutting, rooted plantlets were transplanted into Sunshine Germinating Mix 3 soil (Sun Gro Horticulture Canada Ltd., BC) and fertilized once every two weeks with 100 mL of soluble all purpose Plant-Prod 20–20–20 fertilizer (4 g / L water) (Plant Products Co. Ltd., Nu-Gro IP Inc., Brantford, ON). Seven days after transplanting the cuttings, the photoperiod was changed from eight to 18 hours to induce normal development through the physiological phases.

4.2.2 Comparison of propagation methods for disease assessment across lines

Eight lines with varying levels of resistance to *C. truncatum* were assessed (Table 4.1). The *L. culinaris* lines evaluated were highly resistant LR59-81; partially resistant VIR421, 3155S-5, 'CDC Redberry', and 'Indianhead'; and susceptible 'Eston' and Yerli Kirmizi. One resistant *Lens ervoides* accession (L-01-827A) was included in the survey, as it is the source of resistance for LR59-81 to the highly virulent race Ct0 (Fiala et al., 2009). Comparisons were made between disease ratings of cutting- and seedling-derived plants grown from a single seed of the same line. Seeds were planted in 10-cm diameter pots at the same time cuttings were taken. The

experiment used a randomized complete block design with four replications of each line generated by both seedlings and cuttings.

About one month after cuttings were taken, inoculation with *C. truncatum* was performed on all treatments and replications. At inoculation, all lines of seedling-derived plants except 3155S-5 and 'Indianhead' were in the reproductive phase; 'Eston', LR59–81, and L-01–827A were in full bloom; and VIR421,Yerli Kirmizi, and 'CDC Redberry' had buds but no open flowers. The lines 3155S-5 and LR59-81 were the only cutting-derived plants not in the reproductive phase on inoculation. Cutting-derived plants of 'Eston', VIR421, Yerli Kirmizi, 'CDC Redberry', and 'Indianhead' had buds but not flowers. L-01-827A was the only line of cutting-derived plants in full bloom. On average, development of plants propagated from seed was more advanced than plants propagated from cuttings.

Inoculation with *C. truncatum* isolate 95A8 (ID # CT-34, race Ct0) was conducted at both low (4×10^4 spores / mL) and high (5×10^5 spores / mL) concentrations. Spores were retrieved from cultures grown in 5-cm diameter Petri dishes with oatmeal agar (30 g blended quick oats [The Quaker Oats Company, ON] and 8.8 g agar [Difco, Becton, Dickinson and Company, MD], 1 L deionized H_2O) for seven days. Plants were inoculated with an airbrush sprayer (Badger Mini Spray Gun Set, Model 250.4, Badger Air-Brush Co., IL) at 140 kPa with approximately 15 mL of suspension. For 24 hours post-inoculation, plants were incubated at 100% relative humidity.

Disease ratings were performed 20 days postinoculation for the percentage of the main stem covered in lesions (SL), the percentage of leaves affected by disease including defoliation due to disease (LL) using the scale described by Horsfall and Barratt (1945) as described in section 3.2.3, and the percentage of shoots that had died back due to disease (SDB). Data analyses for low and high spore concentrations were performed separately. Levene's test for homogeneity of variance (Levene, 1960) was applied to factors across all components (SL, LL, SDB). Heterogeneity in SDB across propagation method (cuttings or seedlings) was corrected by requesting residuals for each of the lines in the Mixed procedure in SAS (SAS Institute, Cary, NC) for analyses at both high and low spore concentrations. Similarly, heterogeneity across

lines at low spore concentration for SL was corrected by using non-pooled residuals for testing significance of different factors. Correcting for heterogeneity across lines was not possible in any other analyses using either transformations or non-pooled residuals. The significance of fixed effects (lines and propagation method) was tested in the Mixed procedure in SAS with blocks being a random factor. Least square means, standard errors, and *p*-values for differences between pairs of means were also calculated using the PDIFF option. In addition, Spearman correlations were performed for each of the traits across all lines on the basis of the individual values in SAS.

4.2.3 Evaluation for disease resistance of a segregating population

To compare propagation methods using a segregating F_2 population, 35 F_2 plants from the LR64 population (as described in section 6.2.1), parental lines, and susceptible check 'Eston' were grown as mother plants from which eight cuttings per plant were taken. One week after cuttings were taken, seedlings of F_2s, parents, and checks were seeded into 50-cell trays (75 mL / cell with a 4-cm diameter). Each experimental block contained two trays, one with a cutting treatment of one of each of the 35 F_2 cutting-derived plants and five cutting-derived plants of each parental and check line, and the other tray with a different set of 35 seedling-derived F_2s and five seedling-derived plants of each parent and the susceptible check. The blocks were arranged in a randomized complete block design with four replications. The entire experiment was repeated once.

Before inoculation, the plant height, number of nodes, number of shoots, number of flowering nodes, and number of nodes with pods for plants derived from both cuttings and seedlings were recorded. These traits were analyzed as generalized linear models with normal distribution in the Genmod procedure in SAS. In addition, the proportion of reproductive plants for the three lines and across the F_2 population, comparing the two propagation methods in each block, was analyzed as a generalized linear model with a binomial distribution and logit link function.

Inoculation and disease rating procedures were the same as described above; however, a single concentration of *C. truncatum* was used (10^5 spores / mL) and

disease rating occurred 14 days post-inoculation. When disease scores were tested for heterogeneity of errors, the lines and population were found to be heterogeneous for all parameters assessed (SL, LL, and SDB). Propagation method (cuttings compared to seedlings) was heterogeneous for SDB. Where possible, heterogeneity was corrected by grouping data by line using the Mixed procedure of SAS; however, heterogeneity could not be corrected for SDB. The analysis of variance (ANOVA; first performed in the general linear model [GLM] procedure in SAS) of the checks only as well as the entire data set indicated no significant difference between the experiment replicates, thus data were grouped together for further analysis. 'CDC Redberry', LR59–81, and the susceptible check 'Eston' were first analyzed for differences between seedlings and cuttings using the Mixed procedure in SAS. Least square means were determined in the Mixed procedure for the check and parental lines; *p*-values for differences between pairs of means were also calculated using the PDIFF option. Least square means for each of the 35 replicated F_2 genotypes were determined in a separate analysis.

On the basis of a classification where resistant plants have less than 50% disease and susceptible plants have more than 51% disease, individual F_2s were categorized by disease scores, using the least square means for the cutting-derived replicated F_2s and raw percentage disease scores for the seedling-derived F_2s. Using Chi-square tests, seedling- and cutting-derived F_2s were tested for fit to Mendelian segregation ratios for resistance being controlled by a single dominant gene (3 resistant : 1 susceptible), by duplicate dominant epistasis (15 resistant : 1 susceptible), by duplicate recessive epistasis (9 resistant : 7 susceptible) , and by dominant and recessive epistasis (13 resistant : 3 susceptible). Fit to the different models was assessed by comparing rejection or failure of rejection to each of the models for both cuttings and seedlings. Furthermore, the Genmod procedure using the binomial distribution in SAS was used to test for differences in segregation ratios between cutting and seedling F_2s, and the Poisson distribution in a generalized linear model in SAS was used to test differences in the frequency distribution between seedlings and cuttings.

Owing to the relatively small number of cutting-derived F_2s analyzed (n = 35), results from the seedling-derived plants were compared to a separate experiment where 104 F_2 individuals from the same population were assessed for disease using the cutting method to obtain replications as described in full detail in Chapter 6. In summary, multiple cuttings of individual F_2 plants were taken as described above at regular intervals and rooted, then transplanted, to generate experimental units. Transplanting, inoculating, and rating procedures were the same as used on the F_2 cuttings and seedlings described above except that two blocks were inoculated at once, and the experiment was repeated four times for a total of eight replications. Least-square means of raw disease ratings were calculated using the Mixed procedure in SAS.

4.4 Results and Discussion

Inoculations and disease values for the experiment testing the method across lines showed similar disease to what was expected based on previous results with the susceptible lines with high percentages of lesions or SDB and partially resistant or resistant lines showing lower disease scores. The ANOVA comparing propagation method for disease assessment across lines indicated significant differences between cutting- and seedling-derived plants with respect to both SL ($p = 0.01$) and SDB ($p < 0.01$) at the low inoculant concentration, but no differences between propagation methods based on LL values. The differences in SL and SDB scores were not present in experimental units inoculated with the higher concentration of spores (Table 4.1). When inoculated with the high concentration, very few differences were observed between means of cuttings and seedlings on all lines suggesting resistance mechanisms in resistant lines may have been overwhelmed. An exception was significantly less LL and SDB on cutting-derived plants of 3155S-5 ($p < 0.05$ and < 0.01, respectively; data not shown). At the low spore concentration, however, several significant differences across lines between cuttings and seedlings were evident (Table 4.2). 'CDC Redberry' and 'Indianhead' differed significantly with respect to percentage SL, with the cuttings having less disease. Furthermore, the SDB was significantly less on the cuttings of 3155S-5, 'CDC Redberry', and 'Indianhead'.

Correlations between seedling and cutting ratings were significant, with values of 0.62 ($p < 0.01$) for LL, 0.65 ($p < 0.01$) for SL, and 0.36 ($p < 0.05$) for SDB.

Table 4.1. Variance analysis for propagation method (cutting- vs. seedling-derived plants) on lines of lentil (*Lens culinaris* Medik.) inoculated with *Colletotrichum truncatum*, assessed as the percentage of the main stem and leaves with lesions and shoot dieback.

Spore concentration	Source of variation		Stem lesions		Leaf lesions		Shoot dieback	
		nDf †	dDf ‡	*F* value §	dDf	*F* value	dDf	*F* value
4×10^4 spores/mL	Line	7	14.4	138.78**	48	7.35**	47.3	7.66**
	Propagation method ¶	1	22.6	7.16*	48	3.78	47.3	15.37**
	Line × propagation method	7	14.4	2.20	48	0.79	47.3	1.75
5×10^5 spores/mL	Line	7	46	7.32**	46	7.95**	45.4	5.64**
	Propagation method	1	46	1.42	46	0.15	45.5	2.39
	Line × propagation method	7	46	1.41	46	1.36	45.4	1.43

† Numerator degrees of freedom.

‡ Denominator degrees of freedom, estimated using Kerward-Roger procedure.

§ *$p < 0.05$, ** $p < 0.01$.

¶ Comparing plants grown from seedlings or cuttings.

Both ‘CDC Redberry’ and 3155S-5 have ‘Indianhead’ as part of their pedigree, which may explain the comparable differences in disease ratings of cuttings vs. seedlings. Resistance in ‘Indianhead’ declines as the plant begins to flower (Chongo and Bernier, 2000a), and thus the differences observed in these lines may, in part, be owing to lack of synchronization of development between cutting- and seedling-derived plants. When the correlations of disease ratings between cuttings and seedlings inoculated with a low spore concentration are recalculated without these three lines, the values increase to 0.74 ($p < 0.01$) for SL, 0.75 ($p < 0.01$) for LL, and 0.65 ($p < 0.01$) for SDB.

Table 4.2. Mean disease ratings of the percentage of the main stem covered with lesions and percentage shoot dieback due to infection with *Colletotrichum truncatum* on both cutting- and seedling-derived lentil plants.

Line	Survey across lines (4×10^4 spores/mL)						Evaluation on parental lines and a susceptible check when inoculated with a segregating population					
	Stem lesions (%)			Shoot dieback (%)			Stem lesions (%)			Shoot dieback (%)		
	C†	S†	C vs. S‡	C	S	C vs. S	C	S	C vs. S	C	S	C vs. S
3155S-5	29.69	58.59	28.91 ±18.12	8.25	55.00	46.75 ±22.73*						
Yerli Kirmizi	94.28	98.62	4.34 ±4.25	100.00	100.00	0.00 ±22.73						
CDC Redberry	25.00	81.34	56.34 ±18.88*	25.00	93.75	68.75 ±22.73**	95.35	71.26	24.09 ±4.68**	100.00	62.71	37.29 ±4.17**
LR59-81	5.86	6.44	0.59 ±4.40	0.00	25.00	25 ±22.73	2.60	2.47	0.14 ±0.15	0.00	0.06	0.06 ±4.29
Eston	98.62	94.28	4.34 ±4.18	100.00	100.00	0.00 ±22.73	98.10	96.51	1.60 ±0.11	100.00	99.48	0.52 ±4.20
L-01-827A	22.65	21.09	1.56 ±14.34	24.00	59.50	35.50 ±22.73						
Indianhead	21.09	63.72	42.62 ±16.62*	0.00	74.00	74.00 ±22.73**						
VIR421	58.68	55.86	2.83 ±30.49	56.25	58.25	2.00 ±22.73						
Mean	44.48	59.99	15.51 ±5.79*	39.19	70.69	31.50 ±8.04**						

*Significantly different from zero at $p < 0.05$.

**Significantly different from zero at $p < 0.01$.

† Mean of cutting- (C) or seedling-derived plants (S).

‡ Difference in values between cutting- and seedling-derived plants, standard error of the mean, and significance level.

When mother plants of the eight different lines were grown out at 18-hour days post-cutting, they produced 140 to 830 seeds per plant, which is sufficient seed to pursue further genetic studies in subsequent generations. Based on the results of the initial study, clonal propagation of single F_2 plants held promise as a method to obtain replicated disease evaluation data for *C. truncatum* resistance; however, reasons for the subtle differences in disease levels on some lines need to be explored fully.

In the segregating population, cutting- and seedling-derived plants differed significantly with respect to the number of shoots or branches, the number of flowering nodes, and the number of nodes with pods (ANOVA; data not shown). More shoots were consistently observed on cuttings than seedlings on the basis of pair wise comparisons across the parental and check lines as well as on the F_2 population (Table 4.3). Strigolactones act as a messenger hormone for auxin that directly prevents bud outgrowth inhibiting branching in plants (Dun et al., 2009). It is thought that during the process of rooting cuttings, auxin signalling was compromised inhibiting strigolactones which reduced the apical dominance exhibited by plants grown from seed. Cutting-derived plants significantly differed in the number of nodes compared with seedlings on each line; however, they varied as to which plant type had more. In contrast to the survey across lines, the results of this experiment suggest cuttings may have been developmentally older than seedlings, as each line had significantly more nodes with reproductive structures and nodes with pods. However, the number of plants in the adult phase was the same across the two treatments on the basis of analysis using the binomial distribution (data not shown). With the exception of 'Eston', cutting- and seedling-derived plants were not significantly different in height (Table 4.3).

Table 4.3. Mean number of shoots, nodes, flowering nodes, nodes with pods, or height of cutting- or seedling-derived plants on three lentil (*Lens culinaris*) lines and a segregating LR64 F_2 population.

Line	Shoots (n)			Nodes (n)			Flowering nodes (n)			Nodes with pods (n)			Height (cm)		
	C †	S †	C vs. S ‡	C	S	C vs. S	C	S	C vs. S	C	S	C vs. S	C	S	C vs. S
LR59-81	5.4	1.8	3.6 ±0.4**	15.0	13.5	1.5 ±0.5**	5.5	3.4	2.1 ±0.5**	2.3	0.5	1.9 ±0.4**	30.0	28.5	1.6 ±2.4
Eston	6.5	1.6	4.9 ±0.4**	13.1	14.7	1.7 ±0.5**	5.6	2.7	2.9 ±0.5**	2.7	0.1	2.5 ±0.4**	26.9	36.4	9.5 ±2.3**
LR64 F_2 population	4.3	1.5	2.8 ±0.2**	14.5	14.0	0.5 ±0.2**	7.6	3.2	4.4 ±0.2**	4.1	0.4	3.7 ±0.1**	35.4	35.4	0.0 ±0.9
CDC Redberry	4.8	1.5	3.3 ±0.4**	12.7	13.8	1.1 ±0.5*	6.9	1.3	5.5 ±0.5**	3.7	0.0	3.7 ±0.4**	30.2	30.3	0.1 ±2.3
Total	5.2	1.6	3.6 ±0.2**	13.8	14.0	0.2 ±0.2	6.4	2.7	3.7 ±0.2**	3.2	0.2	3.0 ±0.2**	30.6	32.6	2.0 ±1.0

*Significantly different from zero at $P < 0.05$.

**Significantly different from zero at $P < 0.01$.

† Mean of cutting- (C) or seedling-derived plants (S).

‡ Difference in values between cutting- and seedling-derived plants, standard error of the mean, and significance level.

Data for disease levels of cutting- and seedling-derived plants for the resistant parent LR59-81 and susceptible check 'Eston' were consistent with the initial experiment of this study, with no significant difference between cuttings and seedlings for all disease parameters assessed. Significant differences between disease levels on cuttings and seedlings were observed only on 'CDC Redberry'; in contrast to the initial experiment, cuttings had significantly more disease than seedlings (Table 4.2). In the first experiment, differences observed in lines with 'Indianhead' in the pedigree were attributed to lack of growth phase synchronization. The results of the second experiment are consistent with developmental staging of the plants between the treatments and support this assumption. In the *C. higginsianum-A. thaliana* pathosystems, detaching leaves triggered senescence pathways that interfere with disease response causing false compatible reactions (Liu et al., 2007). Not only may differences in the disease reaction observed on partially resistant lines have been due to differences in development but they have been enhanced by interference with the ethylene pathway when the cuttings were taken. When this method was proposed, the length of time between the initial propagation of the plant to the time of inoculation was presumed to be sufficient to restore these pathways to full capacity. Our results suggest the reaction of lines with partial resistance may be due to residual effects of the cutting procedure; however, the genetic tools to easily test this in *L. culinaris-C. truncatum* pathosystems do not currently exist.

Comparison of frequency distributions of disease scores of the F_2 genotypes using the Poisson distribution indicated significant differences between cuttings and seedlings (data not shown). A clear bimodal distribution was present for all parameters (SL, LL and SDB) in the seedlings. However, the distinction between susceptible and resistant genotypes was not as clear in the cuttings; the lower end of the SDB scale was skewed toward higher percentages in the cuttings compared with the seedlings (Table 4.4). Consistency in scoring LL between cutting- and seedling-derived plants compared with the other disease parameters suggests that this evaluation may more reliably predict resistance when the clonal propagation protocol is used (Table 4.1). However, this parameter is often skewed toward susceptibility in highly resistant lines (data not shown) and did not as clearly differentiate segregating

population into susceptible and resistant classes as the other parameters assessed, especially for the cutting-derived F_2 genotypes (Table 4.4).

Table 4.4. Comparison of frequency distributions of seedling- and cutting-derived F_2s from LR64 inoculated with *Colletotrichum truncatum* for percentage of main stem covered with lesions, percentage of leaves affected by disease, and percentage shoot dieback.

	Stem lesions				Leaf lesions				Shoot dieback			
	Count		Percentage[†]		Count		Percentage		Count		Percentage	
Disease (%)	S[‡]	C[‡]	S	C	S	C	S	C	S	C	S	C
0	0	0	0	0	0	0	0	0	174	5	66	14
1–25	200	17	75	49	163	7	62	20	18	9	7	26
26–50	8	8	3	23	25	8	9	23	28	9	11	26
51–75	8	1	3	2	20	11	8	31	3	3	1	9
76–100	49	9	18	26	57	9	22	26	42	9	16	26
Total	265	35	100	100	265	35	100	100	265	35	100	100
Average Disease[§]	24.44	40.51			36.27	54.22			22.02	41.84		

[†] The count as a percentage of the total number of F_2 genotypes.

[‡] Count or percentage of cutting- (C) or seedling-derived (S) plants.

[§] Average across all seedlings (n = 265) and of F_2s over 8 cutting replications (n = 35).

Binomial analysis comparing the number of F_2 genotypes classified as resistant or susceptible indicated a significant difference between cuttings and seedlings ($p < 0.01$) for all parameters assessed (data not shown). On the basis of SL for seedlings vs. cuttings from the same experiment, there was failure to reject models for genetic control by a single dominant gene (3 resistant : 1 susceptible) or by dominant and recessive epistasis (13 resistant : 3 susceptible), and the model for duplicate dominant epistasis (15 resistant : 1 susceptible) was consistently rejected ($p < 0.05$) (Table 4.5). Differences in Chi-square significance between cuttings and seedlings rated for SDB assessed for fit to the single dominant gene model may be because of differences in original shoot numbers being assessed, as the cuttings tended to have more shoots than plants grown from seed (Table 4.2). On the basis of these differences and the lower

correlation observed for this parameter in the initial experiment of this study, SDB due to disease should be interpreted with caution and not independently as an assessment of resistance when using the cutting method to generate replications. Discrepancies between data from cuttings and seedlings for the duplicate recessive epistasis model (9 resistant : 7 susceptible) were observed for all parameters (Table 4.5) and attributed to the small sample size (n = 35) for the cuttings. A Chi-square test on replicated F_2s (n = 104) from the same population, tested in a different experiment, rejected the null hypothesis of duplicate recessive epistatic control of resistance and was thus consistent with the seedling disease reactions observed in this experiment.

Table 4.5. Cutting- and seedling-derived LR64 F_2 population Chi-square test scores for genetic control of resistance ratios inoculated with *Colletotrichum truncatum* for percentage of (i) the main stem covered in lesions, (ii) leaves affected by disease, and (iii) shoot dieback.

		Single dominant (3:1 †)		Duplicate dominant (15:1)		Dominant and recessive (13:3)		Duplicate recessive (9:7)		
		S ‡	C ‡	S	C	S	C	S	C	P†
	N §	265	35							104
Stem lesions	Ratio ¶	208:57	25:10							76:28
	χ^2 #	1.59	0.12	112.4	30.74	0.99	1.73	53.51	2.79	11.64
	P ++	0.21	0.73	<0.01	<0.01	0.32	0.19	<0.01	0.09	<0.01
Leaf lesions	Ratio	188:77	23:12							31:73
	χ^2	2.17	1.27	248.86	48.55	17.02	4.73	23.42	0.98	27.92
	P	0.14	0.26	<0.01	<0.01	<0.01	<0.05	<0.01	0.32	<0.01
Shoot dieback	Ratio	220:45	24:11							71:33
	χ^2	8.77	0.54	56.22	39.14	0.63	3.04	77.44	1.77	5.87
	p	<0.01	0.46	<0.01	<0.01	0.43	0.08	<0.01	0.18	<0.05

† Ratio for genetic model being tested.

‡ Results for cuttings (C), seedlings (S), or cuttings of the population (P) tested separately.

§ Number of F_2 genotypes analyzed.

¶ Resistant : Susceptible, with resistant genotypes having ≤50% disease.

χ^2 value of test for respective ratio.

++ Probability of a greater χ^2 value under the null hypothesis of the ratio tested with Yates correction for continuity.

4.5 Conclusions

Based on these results, clonal propagation of individual lentil plants seems to be a feasible method for generating replicated ratings for reaction to infection by *C. truncatum* . The seedling treatment resolved the population into more discrete classes in the frequency distributions, indicating the cutting method may distort the results. However, clear classes of resistant and susceptible genotypes are still evident with the cuttings (Table 4.4) for SL and SDB, and segregation of individuals using these parameters are consistent with reactions in seedlings of the same population. Results on both seedling- and cutting-derived F_2 ratings suggest the resistance to race Ct0 of *C. truncatum* in LR59-81 derived from *L. ervoides* is controlled by a major single dominant gene.

Considering the interference reported in the disease-signalling pathway when detaching leaves in the *C. higginsianum* pathosystem on *A. thaliana* (Liu et al., 2007), the authors initiated these experiments, as they felt a detached leaf assay was not suitable for phenotyping resistance to *C. truncatum* in lentil. Furthermore, a previous pilot study testing a detached leaf assay for resistance to *C. truncatum* on lentil could only differentiate highly susceptible 'Eston' from partially resistant lines but did not reflect differences in resistance between highly resistant LR59-81 and partially resistant VIR421 (Matlock et al., personal communication). Comparison of cutting-derived plants in the initial experiment of this study showed consistent significant differences between LR59-81 and VIR421 (data not shown). Thus, although not ideal, this propagation method provides an attractive alternative by generating replicates from a single plant compared with using detached leaves to assess disease. Furthermore, the propagation method may also be able to reveal the complexity of a trait as it amplifies subtle differences between lines or genotypes.

Results of this study suggest disease evaluation may yield misleading results if SDB on cuttings inoculated with *C. truncatum* is the only parameter assessed. The results from this particular F_2 population also suggest this method may be more appropriate in a population where age-dependent resistance is not involved. Even

given this complexity, the results will still be useful when supported by the phenotypic characterization, if required, of the segregating F_3 families. The replication generated via the propagation protocol allows a cushion for failed inoculations that are common with this pathogen; replications are needed to overcome the variability introduced with larger experiments when testing larger population sizes. Furthermore, use of this method allows for development of F_3 families from each F_2 plant and subsequent development of RILs. Information from the earlier generation may indicate if further investment and continued development of the population is warranted to investigate complex traits. The ability to test specific F_3 families based on a replicated F_2 disease resistance phenotype and correlation of results between generations may prove to be a valuable tool in deciphering the genetics of this particular pathosystem.

4.6 Prologue to Chapter 5

It was concluded that in testing for resistance to *C. truncatum* in lentil, the single-plant replication by propagation protocol provided the best option to obtain sound phenotypic data. However, results from the study described in Chapter 4 and early results in utilizing the protocol to test for resistance in a segregating population (Appendix 14 and 20) showed the confounding results due varying flowering dates. It was decided that in order to properly phenotype a segregating population for resistance where 'CDC Redberry' was a parent, a better understanding of the effect of plant age on disease resistance was needed. Thus, the experiments described in Chapter 5 were initiated.

CHAPTER 5

THE EFFECT OF PLANT AGE ON RESISTANCE TO *COLLETOTRICHUM TRUNCATUM* IN *LENS CULINARIS*

5.1 Introduction and Objectives

Various crop species have shown stage, phase or age-specific expression of disease resistance. This is considered a limiting factor in maximizing production thus represents an important target for improvement of genetic resistance (Whalen, 2005). However, there is little understanding of the relationship between plant development and disease resistance (Chintamanani et al., 2008; Develey-Rivière and Galiana, 2007). Developmental resistance has been defined as gradual or abrupt changes in resistance to a pathogen that are correlated to the developmental stage of the host or of plant organs (Whalen, 2005). Most common is resistance that is acquired with age, often called age-related resistance (Develey-Rivière and Galiana, 2007). Seedling resistance can sometimes be effective for all phases of the plant life-cycle as has been found for *Xanthomonas oryzae* pv. *orzae* rice resistance genes (Mew, 1987).

Seedling, vegetative or juvenile phase (JP) resistance that decreases as the plant ages has also been reported from various pathosystems, however is not as common or as well studied as age related resistance. For example, resistance to spot blotch in wheat progressively decreases between the completion of anthesis and the late milk grain stage due to increased sensitivity to the toxin helminthosporol produced by *Cochliobolus sativus* (Vergnes et al., 2006). Powdery mildew seedling resistance that is absent in the adult phase (AP) has been reported in pepper (de Souza and Café-Filho, 2003), similar to spot blotch resistance in barley (Bilgic et al., 2006). Furthermore, resistance to turnip mosaic virus in Chinese cabbage has also shown a similar resistance pattern with age (Zhang et al., 2008).

Resistance to anthracnose caused by *Colletotrichum graminicola* in sorghum and corn has also been shown to decrease with plant development (Ferreira and Warren, 1982; Jamil and Nicholson, 1987; Ngugi et al., 2000; Nicholson et al., 1985). A previous study on lentil examined the effect of inoculating plants at different growth stages of three lines ('Eston', 458-7 and 'Indianhead'), that vary in the level of resistance to anthracnose with an isolate of race Ct1 of *Colletotrichum truncatum* (Chongo and Bernier, 2000a). The study concluded that seedling resistance decreases at the 8-10 node and then is somewhat re-acquired through the flowering and early pod stages, regardless of the resistance level to the disease.

Initial experiments of this project were continually confounded by the effect that plant age had on expression of resistance in lines such as 'CDC Redberry'. For example, the population LR64 (described in 6.2.1) proved to be highly problematic to phenotype for disease resistance as it was also segregating for time to flowering which had a profound impact on disease resistance classification. This was first observed when testing the cutting protocol on a segregating population (as described in Chapter 4) then later on when the first attempt to phenotype LR64 F_2s was initiated (Chapter 6 and Appendix 14 and 15). It was evident that in order to: i) carefully plan experiments to phenotype the segregating populations; ii) clarify the results of various studies of this project; and iii) gain a better understanding of the phenomenon for future studies, in-depth investigation was needed.

The purpose of the current study was to evaluate phase-dependent resistance to the more virulent race Ct0 of *C. truncatum* in lentil lines with new sources of resistance identified in the *Lens culinaris* Medik. gene pool as well as resistance derived from *L. ervoides*. The initial study concluded that the line 'CDC Redberry' showed differential response to the pathogen in the JP versus the AP. Based on these results, a second study was employed that followed the decrease in resistance in 'CDC Redberry' plants over several stages and comparing it to a susceptible and a resistant line. Resistance was acquired after the seedling stage, but then decreased through progression of reproductive development. These results conflicted with the observations of Chongo and Bernier (2000a) and the differences were hypothesized to be due to the effect of the more virulent race Ct0 of the pathogen used in the study.

To resolve this, a final experiment was conducted to evaluate resistance of selected lentil genotypes inoculated lines at different ages with both races of *C. truncatum* . This experiment found resistance decreased through the AP on all partially resistant lines with significant differences being greater when inoculated with race Ct0.

5.2 Materials and Methods

5.2.1 Fungal isolates

Spores of *C. truncatum* isolate 95A8 (ID # CT-34, race Ct0) or 95B36 (ID # CT-35, race Ct1) were grown and harvested as described in section 4.2.2.

5.2.2 Plant material and inoculation

The susceptible checks were Yerli Kirmizi and 'Eston', partially resistant lines were 'CDC Redberry', 'Indianhead', VIR421 and breeding line $F_{2:6}$ 3155S-5 and the resistant lines were L-01-827A and the interspecific recombinant inbred line (RIL) LR59-81 (Table 2.1 and section 1.1). 'CDC Robin' has resistance to race Ct1 but is susceptible to race Ct0 infection (unpublished data) and was included in one of the experiments.

Planting, inoculations and disease ratings were performed as described in section 4.2.2 with minor modifications. Three plants of each line were grown per pot constituting a replicate of a treatment in 18 hour photoperiod. Disease ratings for stem lesions (SL) and shoot die back (SDB) taken 21 days post-inoculation were used for analysis except for the experiment that examined resistance progression for 'CDC Redberry' for where ratings taken 14 days post-inoculation were used for analysis. Ratings of previous experiments had shown ratings for the pathogen on these lines at 14 and 21 days post-inoculation were almost identical (data not shown).

5.2.3 Experimental Design

5.2.3.1 Survey of resistance at juvenile and adult phases across lines

For a preliminary survey of resistance at the JP and AP growth phases, the eight lentil lines (Yerli Kirmizi, Eston, 'CDC Redberry', 'Indianhead', VIR421,

3155S-5, L-01-827A and LR59-81) with differing levels of resistance and / or source of resistance were compared. The AP plants were inoculated with the representative isolate of race Ct0 six weeks after planting and all lines were developing pods except for 'Indianhead' which had only buds. Despite the range in the stage of reproduction between lines of the AP, they were still considered one treatment as all plants were in the adult vegetative stage as described by Poethig (1990). Seeds for JP plants were planted three weeks prior to inoculation with the same spore suspension and had five to nine nodes, none of which had reproductive structures. The experimental units were arranged in a randomized complete block design with four replicates. A concentration of $5x10^5$ spores / mL was sprayed on experimental units; approximately 20 mL of suspension on AP plants and 5 mL of suspension on JP plants. The experiment was performed in the summer of 2006. In subsequent experiments, the spore concentration used for inoculations was lower as conditions were still being optimized when the preliminary experiment was performed.

5.2.3.2 Resistance progression in 'CDC Redberry'

To analyze the progress of resistance decline in 'CDC Redberry' at various stages of development in more detail, plants were inoculated with race Ct0 19, 26, 33, 40 and 47 days after planting along with the susceptible check 'Eston' and the resistant check LR59-81. The number of nodes and the number of flowering nodes was recorded on each individual plant immediately before inoculation (Table 5.1). 'CDC Redberry' and 'Eston' plants were at the eight to ten node stage 19 days after planting. The early flower stage occurred at approximately 33 days after planting, and plants were developing pods at 47 days. LR59-81 started flowering approximately seven days earlier than 'CDC Redberry' and 'Eston'. The experimental design was the same as previously described except the entire experiment was repeated twice. Inoculations were performed as described earlier except that a concentration 10^5 spores / mL of *C. truncatum* was used. Both repeats of the experiment were performed in the summer of 2008.

Table 5.1. The mean number of nodes and the mean number of nodes with reproductive structures on lentil lines at different ages.

Age	Number of Nodes			Number of Reproductive Nodes		
	LR59-81	Eston	CDC Redberry	LR59-81	Eston	CDC Redberry
19 days	7	8	10	0	0	0
26 days	10	11	11	2	0	0
33 days	13	12	13	5	1	1
40 days	16	16	16	6	4	4
47 days	17	18	17	8	4	4

5.2.3.3 Effect of race of *Colletotrichum truncatum* at different ages

To examine the effect of race of *C. truncatum* at key growth stages of plants associated with resistance decline identified in the first two experiments, plants of 'Eston', LR59-81, 'CDC Redberry', 'CDC Robin' and VIR421 were inoculated 19, 26 and 47 days after planting with the representative isolates of race Ct0 or race Ct1 at a rate of 10^5 spores / mL. The experiment was performed in the fall of 2008 and was arranged as a randomized complete block design with eight replicates within a single experiment.

5.2.4 Statistical analysis

All analyses were conducted in SAS (SAS Institute, Cary, NC). Levene's test for homogeneity of variance (Levene, 1960) was used to test variances among SL and SDB ratings. Based on the factors that had heterogeneous variances, corrections were made by remodelling the SAS program to request multiple residuals for testing significance using the repeated statement in the Mixed procedure or by using the arcsine transformation. Repeats of the experiment (where appropriate), blocks within repeats and repeat by treatment interaction effects were considered random factors. Covariance parameter estimates indicated that the variance of the experimental repeats and repeat by treatment interactions for the second experiment accounted for a low

proportion of the overall variation in the experiment studying resistance decline over the life-span of the plants. Thus, data from repeats of the experiment were pooled for further analysis. Least square mean differences, standard errors and *p*-values for comparison of means were calculated using the PDIFF option in SAS.

5.3 Results

When the results of all three experiments were compared, it was evident 'CDC Redberry' showed the greatest variation in disease response depending on the age and growth-phase of the plants. Regardless of the amount of infection, the disease ratings on this line were most consistent between experiments as the plants became older, developing a highly susceptible reaction at six weeks after planting when the plants were in the early podding stage. Given the lower spore concentration of 10^5 spores / mL of *C. truncatum*, the interspecific RIL LR59-81 showed a high level of resistance that was relatively unaffected by age or growth phase. VIR421 also had high levels of resistance, however, was prone to resistance decline with increasing age, especially in the preliminary experiment. The susceptible check 'Eston' had significantly greater disease than partially resistant and resistant lines.

5.3.1 Survey of resistance at juvenile and adult phases across lines

Infection by *C. truncatum* race Ct0 in the preliminary experiment was quite high across all lines, most likely due to the excessive spore concentration used in the inoculation. In later experiments, the spore concentration was reduced to better differentiate between resistant and susceptible lines. However, differences between the treatments were still observed in this preliminary experiment as the phase, the line, and the phase by line interaction all had significant effects on SL and SDB ($p < 0.05$).

It was found when eight lines were surveyed for resistance over JP and AP, three general resistance levels were evident. Yerli Kirmizi, 'Eston' and 'Indianhead' were susceptible, L-01-827A and 'CDC Redberry' were partially resistant and LR59-81, 3155S-5 and VIR421 were resistant. The line VIR421 had the highest level of resistance under high disease pressure. Differences between the JP and AP plants were observed consistently between parameters on 3155S-5, 'CDC Redberry' and

VIR421 (Table 5.2). When the SDB was compared, JP plants of LR59-81 also had significantly less disease. For both disease assessment parameters 'CDC Redberry' and VIR421 showed the greatest degree of difference in disease increase in response to growth phase compared to all other lines.

Table 5.2. Comparison lines of lentil at the juvenile and adult phases inoculated with $5x10^5$ spores / mL of *Colletotrichum truncatum* race Ct0 for the percentage of the stems covered in lesions and percentage shoot die-back

	Stem Lesions (%)			Shoot Die-Back (%)		
Line	**JP** †	**AP** †	**JP vs. AP** ‡§	**JP**	**AP**	**JP vs. AP** §
3155S-5	37.15	58.93	21.78±9.46*	46.04	80.33	34.28±8.95**
Yerli Kirmizi	98.62	98.62	0.00±9.23	100.00	100.00	0.00±8.80
CDC Redberry	66.84	93.25	26.41±9.23**	64.31	97.22	32.92±8.80**
LR59-81	42.36	61.38	19.01±10.02	61.81	81.24	19.44±9.33*
Eston	98.62	95.45	3.17±9.23	100.00	100.00	0.00±8.80
L-01-827A	86.01	75.39	10.62±10.39	95.83	100.00	4.17±9.57
Indianhead	95.61	98.07	2.45±9.23	95.83	100.00	4.17±8.80
VIR421	16.41	45.71	29.31±9.46**	2.78	44.13	41.36±8.95**

† Mean disease of plants at juvenile phase (JP) (inoculated 21 days after planting) and adult phase (AP) (inoculated 42 days after planting).

‡ Difference in values between JP and AP, standard error of the mean and significance level.

§ Significant differences tested based on data grouped by phase to adjust for heterogeneous errors.

* Significantly different from zero at $p < 0.05$; ** Significantly different from zero at $p < 0.01$.

The line 3155S-5 had significantly less disease than 'CDC Redberry' and 'Indianhead' ($p < 0.05$) at both phases suggesting improved resistance under high disease pressure. However resistance in this line appears to decline in the same pattern as 'CDC Redberry'. Similar disease levels were observed in LR59-81 as in 3155S-5 in the preliminary experiment. However, resistance in LR59-81 appeared to be the relatively stable over both growth phases compared to the other resistant lines and had improved resistance over the parent L-01-827A in the JP for stem lesions and in both phases when the shoot die-back was analyzed ($p < 0.05$). Accession VIR421

showed moderate resistance that was greater than both 3155S-5 and LR59-81 for stem lesions in the JP and had reduced SDB at both phases ($p < 0.01$), but was also vulnerable to decrease in the AP.

The results suggested that phase-dependent resistance to race Ct0 of *C. truncatum* may be consistent with results for those reported by Chongo and Bernier (2000a) for race Ct1. However the magnitude of differences in disease severity between JP and AP observed for 'CDC Redberry' warranted further investigations into the decline of resistance.

5.3.2 Resistance decline in 'CDC Redberry'

It was found the susceptible check 'Eston' had near-maximum disease levels throughout the life-cycle of the plant where the resistant check LR59-81 showed very low levels of disease regardless of plant age. These results suggested inoculation procedures and environmental conditions had been optimized for this experiment. The substantially lower disease levels on LR59-81 in this experiment compared to results observed in the preliminary experiment is most likely due to the five-fold reduction in spore concentration used. As anticipated, 'CDC Redberry' showed varying levels of disease at various ages throughout development.

When the disease ratings at the five different plant ages were examined by analysis of variance, it was found that all fixed factors were highly significant ($p < 0.0001$) indicating differences among lines at all ages. Highly resistant LR59-81 did not show significant differences in disease in response to plant age. Disease levels on the highly susceptible line 'Eston' decreased slightly at 26 days and did not stabilize until the plants started to flower at 33 days after planting (Table 5.3). 'CDC Redberry' had large significant differences in disease based on both SL and SDB in response to plant age compared to the other lines. Moderate susceptibility was observed on 'CDC Redberry' on the youngest plants at 19 days followed by a decrease in disease between 19 and 26 days. This suggests resistance is acquired as the plant progresses through the JP. There was a decrease in resistance in 'CDC Redberry' between 33 and 47 days as the plants progressed through the AP. This overall trend was also evident at a lower magnitude in the susceptible check 'Eston'.

Table 5.3. Disease on lentil lines throughout the plant life-cycle inoculated with 10^5 spores / mL of *Colletotrichum truncatum* race Ct0 evaluated for the percentage of the stems covered in lesions and percentage shoot die-back.

	Line	19 d †	26 d	33 d	40 d	47 d	19d vs. 26d ‡	26d vs. 33d	33d vs. 40d	40d vs. 47d
Stem Lesions (%)	LR59-81	3.7	3.2	3.0	2.5	2.6	-0.5±0.4	-0.2±0.4	-0.5±0.4	0.0±0.4
	Eston	98.5	93.2	98.5	96.4	96.1	-5.3±1.6**	5.3±1.6**	-2.1±1.6	-0.2±1.6
	CDC Redberry	74.6	49.9	51.1	88.2	94.1	-24.7±5.5**	1.1±5.6	37.1±5.6**	5.9±5.6
Shoot Die-Back (%)	LR59-81	0.0	0.8	0.0	0.0	0.0	0.8±0.5	-0.8±0.5	0.0±0.5	0.0±0.5
	Eston	100.0	86.0	100.0	100.0	100.0	-14.0±4.1**	14.0±4.0**	0.0±4.1	0.0±4.1
	CDC Redberry	68.2	23.8	12.3	74.1	97.1	-44.4±8.1**	-11.5±8.2	61.9±8.2**	23.0±8.2**

† Mean disease over plants 19, 26, 33, 40 and 47 days old.

‡ Difference in disease rating values in the respective ages, standard error of the mean and significance level.

* Significantly different from zero at $P < 0.05$; ** Significantly different from zero at $P < 0.01$.

5.3.3 Effect of race of *Colletotrichum truncatum* at different ages

Disease severity on resistant LR59-81 was similar in this experiment to the previous experiment examining resistance levels of 'CDC Redberry' throughout the life cycle. However, disease levels on 'Eston' and 'CDC Redberry' were less than the previous experiment, especially in the 19 day old and 26 day old plants. Differences between the two races of *C. truncatum* were evident on partially resistant lines with race Ct0 causing higher levels of disease on 'CDC Redberry' and 'CDC Robin'. Resistance in both LR59-81 and VIR421 was relatively unaffected by both race and the age of the plants. The susceptible check had higher disease levels than all other lines at all ages and with both races, however full disease potential was not realized except on the oldest plants, presumably due to the effects of resistance associated with the JP.

All factors (line, age and race) and interactions had highly significant effects on both SL and SDB stem lesions except for the age by race interaction on the SL measurements. Significant differences in disease ratings between different ages, regardless of *C. truncatum* race, were most pronounced for 'Eston', 'CDC Redberry' and 'CDC Robin' (Table 5.4). Lesions covering the stems and SDB symptoms seemed to affect 'Eston' at an earlier age when inoculated with race Ct0 compared to race Ct1. Response to race Ct0 was similar across ages on 'CDC Redberry' and 'CDC Robin'. However, when inoculated with the less virulent race Ct1, 'CDC Redberry' had much larger increase in disease in JP plants at 19 and 26 days old versus podding plants at 47 days old compared to 'CDC Robin'. Significant differences were detected on LR59-81 and VIR421 across age treatments, but the magnitude of changes suggest that resistance in these lines is much less age-dependent compared to the other lines.

The ability of 'CDC Robin' across all ages to differentially react to race Ct1 and Ct0 of *C. truncatum* was evident as significant differences were observed more consistently and at a higher magnitude in this line than for all other lines (Table 5.4). Although differences between the two races were observed at each plant age of 'CDC Robin', the largest differences were evident when the plant was forming and filling pods at 47 days old (Table 5.4). It is possible that differentiation between the two races is equally effective using younger plants of 'CDC Robin' mid-flower, but this

would require further testing. Significant differences between the races were also observed for SL on 'CDC Redberry' at 26 days, and for both disease parameters at 47 days (Table 5.5). However similarity in the overall trend in these patterns of resistance suggest that the same resistance mechanisms (and genes) are regulating plant response to both races, but that race Ct0 may be better at circumventing plant defence than race Ct1 resulting in more disease (Table 5.4).

Table 5.4. Disease on lentil lines inoculated with 10^5 spores / mL of *Colletotrichum truncatum* races Ct0 and race Ct1 at various ages evaluated for the percentage of the stems covered in lesions and percentage shoot die-back

Line	Race Ct0					Race Ct1				
	19 d [†]	26 d	47 d	19d vs. 26d [‡]	26d vs. 47d	19 d	26 d	47 d	19d vs. 26d	26d vs. 47d
Stem Lesions (%) [§]										
Eston	41.5	84.9	96.2	43.5±4.2**	11.3±3.7	44.0	66.0	98.2	22.0±4.2**	32.3±3.7**
LR59-81	3.0	1.8	4.8	-1.2±4.3*	3.1±3.8**	1.7	1.7	2.8	0.0±4.2	1.2±3.9**
CDC Redberry	24.2	31.1	87.3	6.8±4.2*	56.3±3.7**	17.6	16.9	69.3	-0.7±4.3	52.5±3.8**
CDC Robin	31.3	25.5	93.9	-5.7±4.2	68.3±3.7**	2.8	2.3	5.1	-0.5±4.3	2.7±3.8**
VIR421	12.3	21.1	23.1	8.8±4.2**	2.0±3.8	15.3	21.4	26.8	6.1±4.2*	5.4±3.8*
Shoot Die-Back (%) [¶]										
Eston	21.9	89.4	100.0	67.5±5.8**	10.7±5.1*	36.6	59.7	100.0	23.1±5.8**	40.3±5.1**
LR59-81	0.1	0.0	0.1	-0.1±5.9	0.1±5.2	0.0	0.0	0.2	0.0±5.8	0.2±5.3
CDC Redberry	7.1	4.9	95.3	-2.2±5.5	90.4±5.1**	1.7	3.5	83.2	1.8±5.9	79.8±5.2**
CDC Robin	15.6	13.9	100.0	1.7±5.8	86.1±5.1**	0.1	0.0	13.6	-0.1±5.9	13.6±5.2**
VIR421	4.2	0.0	4.5	-4.2±5.8	4.5±5.2	5.2	0.0	8.2	-5.2±5.8	8.2±5.2

[†] Mean disease over plants 19, 26 and 47 days old at 8-10 node, late juvenile and early pod stages, respectively.

[‡] Difference in disease rating values in the respective ages, standard error of the mean and significance level.

[§] Significant differences between ages determined using arcsine square root transformed data with errors pooled by age of experimental units.

[¶] Significant differences between ages determined using data with errors pooled by age of experimental units.

* Significantly different from zero at $P < 0.05$; ** Significantly different from zero at $P < 0.01$.

Table 5.5. Comparison of disease on lentil lines inoculated with 10^5 spores / mL of race Ct0 versus race Ct1 of *Colletotrichum truncatum* at 19, 26 and 47 days after planting evaluated for the percentage of the stems covered in lesions and percentage shoot die-back

	Stem Lesions (%)			Shoot Die-Back (%)		
Line	**19 d** †	**26 d**	**47 d**	**19 d** ‡	**26 d**	**47 d**
Eston	-2.5±4.4	19.0±4.0**	-2.0±3.5	-14.7±6.1*	29.6±5.5**	0.0±5.7
LR59-81	1.3±4.5	0.1±4.0	2.0±3.6	0.1±6.2	0.0±5.5	-0.1±4.9
CDC Redberry	6.6±4.4	14.2±4.1**	18.0±3.5**	5.4±6.1	-4.8±6.8	12.0±4.7*
CDC Robin	28.4±4.5**	23.2±4.0**	88.8±3.5**	15.6±6.2*	13.9±5.5*	86.5±4.8**
VIR421	-3.0±4.4	-0.3±4.0	-3.7±3.5	-1.0±6.1	0.0±5.5	-3.7±4.8

† Difference in race Ct0 and race Ct1 disease rating values of 19, 26 or 47 day-old plants, standard error of the mean and significance level. Significance of Stem Lesion differences tested using arcsine square root transformed data with errors pooled by age of experimental units.

‡ Difference in race Ct0 and race Ct1 disease rating values at the respective ages, standard error of the mean and significance level. Significance of Shoot die-back differences tested using data with errors pooled by age of experimental units.

* Significantly different from zero at $p < 0.05$; ** Significantly different from zero at $p < 0.01$.

5.4 Discussion and Conclusions

Results observed in the study looking at age-dependent resistance decline on 'CDC Redberry' and 'Eston' may be partially consistent with results observed by Chongo and Bernier (2000a). Pre-flowering plants that were 8-10 nodes from their study (19 day-old plants from this study) had a peak in disease compared to flowering plants in both studies. However, Chongo and Bernier (2000a) found that disease severity decreased with increasing age on these lines up to eight weeks after seeding whereas disease severity continued to increase in this study. Differences observed later in AP 'Eston' plants may have been due to differences in virulence patterns of the different races of *C. truncatum* as the former study inoculated with a race Ct1 isolate and this study used and isolates classified as race Ct0. Thus, it was imperative to

further examine the interaction of race of *C. truncatum* , lines with different resistance and the age of the plant.

However, when the results of the experiment examining the effect of the different races on lines of different ages is considered, disease levels of the common line 'Eston' in response to the different plant ages is not consistent with results observed by Chongo and Bernier (2000a). They observed a decrease in disease as both susceptible and partially resistant lines progressed through the AP when inoculated with an isolate of race Ct1. It is possible that differences in the experimental protocol, such as the higher spore concentration used in the current study (4×10^4 spores / mL versus 1×10^5 spores / mL), and changes in incubation conditions and disease rating system could have contributed to the different results observed in these two studies. A decrease of disease severity between 19 and 26 day-old plants in this experiment are not consistent with observations on the previous experiment that examined only three lines inoculated with race Ct1. The overall lower disease levels in this experiment demonstrate the difficultly in obtaining replicated results on this disease, especially when subtle differences, such as resistance at specific ages, are being examined. It is possible that differences in the inoculation procedures and microclimate could have contributed to differences despite extensive attempts to replicate the experimental conditions. However, the dramatic decrease of resistance on 'CDC Redberry' when inoculated with race Ct0 is consistent between 26 and 47 day-old plants, confirming age-dependent resistance in the JP to the pathogen across both races in this line. The only other study comparing different aged lentil plants inoculated with *C. truncatum* was done by Wang (2009) who found that conidial germination was higher on six week old plants compared to three week old plants, suggesting inhibitory compounds on the plant leaf surface may play a role in the results observed.

The patterns of disease resistance decrease observed in these experiments are consistent with those observed for resistance to other *Colletotrichum* pathogens on other plant species. Resistance acquisition in the JP that decreases in the reproductive stage has been observed on corn infected with *C. graminicola*. Corn plants are susceptible to anthracnose infection as seedlings and then become resistant as the

leaves expand and mature, followed by resistance decline at the onset of anthesis. The second phase of susceptibility is associated with the onset of leaf senescence (Jamil and Nicholson, 1987; Nicholson et al., 1985). Additional field stress, such as nematode infection and water stress, that decrease the time to senescence, have been shown to increase the severity of anthracnose earlier on in the season (Nicholson et al., 1985). Similarly, inducing senescence in *Arabidopsis* causes greater disease severity when infected with *C. higginsianum* suggesting senescence may play a significant role in disease development (Liu et al., 2007). Resistance decline in sorghum to *C. graminicola* is thought to coincide with decreasing production of precursors for manufacturing of hydrogen cyanide, thought to be toxic to the pathogen (Ferreira and Warren, 1982). The legumes lima bean (Ballhorn et al, 2008) and white clover (Majumdar et al., 2004) have been shown to be cyanogenic, however the genus *Lens* has not been documented to possess glucosides or the degradative enzymes involved in the production of hydrogen cyanide. Furthermore, cyanogenesis has mostly been studied in response to herbivory in legumes, but not in relation to pathogen infection.

Symptoms of spot blotch, caused by *C. sativus*, on five wheat lines decreased between the 4-leaf and booting stage then increased through the completion of anthesis, dramatically increasing in the late milk stage. It was suspected that in this pathosystem, susceptibility with increasing age was due to physiological changes associated with leaf senescence and source-sink metabolism. The phytotoxin helminthosporol is produced by *C. sativus* and sensitivity to the toxin in wheat leaves also increased with plant age, especially in susceptible lines (Vergnes et al., 2006). In the *C. sativus*-wheat pathosystem, it is thought that the compounds produced by the pathogen may trigger ethylene production which contributes to leaf senescence which in turn increases infection (Vergnes et al., 2006). Reports of toxin production by *Colletotrichum* species are rare and undocumented in *C. truncatum* , but *C. lindemuthianum* (Fernández et al., 2000) and *C. acutatum* (Moral et al., 2009) have both been found to produce phytotoxins. Callus derived from resistant and susceptible common bean lines showed differential response to filtrates of *C. lindemuthianum* (Fernández et al., 2000) and the toxic activity of two races of *C. lindemuthianum* were different. Toxic substances of *C. acutatum* caused wilting and branch die-back

syndrome in olive and differential response was also seen in two olive lines in response to culture filtrate of *C. acutatum* (Moral et al., 2009).

Before this study was performed, a deeper understanding of age-dependent resistance to *C. truncatum* in lentil was necessary for both lentil researchers and breeders. Results from this study suggest the timings of inoculations for subsequent experiments and resistance screening in breeding programs should be delayed as long as feasibly possible to ensure juvenile resistance genes are not contributing to lower disease levels. Furthermore, 'CDC Redberry' is becoming increasingly popular with growers due to various favourable agronomic characteristics such as lodging resistance and seed type and will appear frequently in the pedigrees of lentil lines that will be released over the next few years from the Crop Development Centre. Results of this study may provide insight into inevitable future epidemics of anthracnose on the Canadian prairies on this line, especially in seasons with abundant late-season moisture and warm conditions. It is currently unknown if manipulation of seeding date could be used as an anthracnose mitigation strategy, but it could be investiguated based on the results of this study. The phenotypes established in this study provide a good starting point for further investigation into JP resistance in this pathosystem, however further testing to confirm seedling susceptibility and testing independent of seedling and adult susceptibility is needed. It is currently unknown if a more diverse collection of race Ct1 resistance germplasm exhibits the same fluctuation in resistance, thus should also be tested. Based on the results of these experiments, genetic control of resistance at the JP and the AP is being elucidated.

5.5 Prologue to Chapter 6

Results from this chapter show that that resistance in the interspecific line LR59-81 is relatively unaffected by the age of the plant. Compared to the susceptible check 'Eston', there was less variability in disease values in LR59-81 between ages in the latter two experiments of this study. This supports investigation of inheritance of JP resistance from 'CDC Redberry' in the resistant background of LR59-81. Results confirmed and defined resistance decline in 'CDC Redberry' as the plant ages and provided a platform for proper experimental design for the experiments performed in

Chapter 6. Furthermore, the results of this study were important for interpretation of the results from the cutting-protocol study (Chapter 4) and the experiments testing genetic control in segregating populations(Chapter 6).

CHAPTER 6

GENETIC CONTROL OF INTERSPECIFIC-DERIVED AND JUVENILE RESISTANCE IN LENTIL TO *COLLETOTRICHUM TRUNCATUM*

6.1 Introduction and Objectives

Resistance to *C. truncatum* was found in several interspecific recombinant inbred lines (RILs) from a cross between *L. culinaris* cultivar 'Eston' and *L. ervoides* accession L-01-827A (Fiala et al., 2009). Results from Chapter 3 indicated field-resistance found in this population was superior to that found in the *L. culinaris* gene pool. When inoculated with both race Ct1 and race Ct0 of *C. truncatum* , the segregation pattern of resistant to susceptible lines in the RILs suggested that resistance to each race was controlled by two recessive genes (Fiala et al., 2009). It was expected that these results were skewed due to segregation distortion due to genomic divergence and incompatibilities between the speicies causing deviations from Mendelian segregation. Thus, resistance in *L. culinaris* backgrounds are being investigated.

In Chapter 5, it was found that the line 'CDC Redberry' possessed higher levels of resistance in the juvenile phase (JP) than in the adult phase (AP) and that resistance seemed to be acquired as the plant progressed through the vegetative phase. This pattern of resistance decrease on 'CDC Redberry' was consistent when inoculated with race Ct1 of *C. truncatum* . The genetics of age-dependant resistance to *C. truncatum* in *L. culinaris* has not been investigated, thus a segregating population with 'CDC Redberry' as a partially resistant parent was used to investigate the underlying genetic control of resistance in the JP versus AP.

Two sets of experiments were performed to study the inheritance of resistance to *C. truncatum* . The first was to determine the inheritance of resistance to race Ct0 and Ct1 in *L. culinaris* derived from *L. ervoides* accession L-01-827A and the second was to study phase-dependent resistance in *L. culinaris*. The overall objectives of the experiments were: i) to determine if genetic control of resistance to *C. truncatum* is conferred by major genes or if the trait is quantitatively controlled; ii) to determine the dominance relationship of resistance in an interspecific *L. culinaris* x *L. ervoides* RIL; iii) to determine the number of genes controlling resistance in the interspecific RIL to the two races of *C. truncatum* by examining segregation of resistance in a susceptible and partially resistant *L. culinaris* background; and iv) to compare the genetic control of resistance in *L. culinaris* between JP and AP plants.

6.2 Materials and Methods

6.2.1 Plant material and population development

The interspecific RIL LR59-81 was previously shown to have high levels of resistance to both races of *C. truncatum* (Fiala et al., 2009). Many of the RILs from LR59, including LR59-81, exhibited partial sterility. Other reported *L. culinaris* x *L. ervoides* interspecific populations have exhibited sterility which has been shown to be due to chromosomal rearrangements between the species (Tadmor et al., 1987). Of six highly resistant LR59 lines from the interspecific RIL population (data not shown) originally selected for crossing, line LR59-81 proved to be the most genetically compatible when crossed with *L. culinaris* (approximately 2% of the crosses resulting in F_1 seeds compared to 0% for the other five lines).

LR59-81 was crossed with 'CDC Redberry' which is partially resistant to *C. truncatum*, and with the susceptible line 'Eston' to generate two introgression populations used to study genetic control of resistance. The LR59-81 x 'CDC Redberry' cross was made in the winter of 2006 and was named LR64. This population was developed to examine genetic control of resistance to both races of *C. truncatum* and to determine genetic control to race Ct0 at juvenile compared to adult phases. The LR59-81 x 'Eston' cross was made in summer of 2006 and was named

LR67. This population was used to study genetic control of the different races only (Table 6.1). Segregating F_2 populations were developed from a single F_1 seed from each cross and consisted of 107 and 114 F_2 individuals for LR64 and LR67, respectively. Additional crosses were performed to obtain more F_1 seeds to directly compare resistance of F_1 plants with F_2 individuals within the same study for race comparison.

In a GR48 growth room (Conviron®, Controlled Environments Limited, Winnipeg, MB), F_1 and F_2 plants as well as parental lines were developed by first growing F_2 mother plants from a single lentil seed in eight hour photoperiod for six weeks (21^{o}C Day : 15^{o}C Night). Single seeds were planted as described in section 4.2.2. Multiple cuttings of the F_1s, F_2 plants and parental lines were taken at regular intervals, were rooted and then transplanted to generate experimental units as described in section 4.2.1. Following removal of cuttings for both studies, each F_2 plant was allowed to self-pollinate and mature to produce seed for the $F_{2:3}$ family.

For both populations, F_2 plants were grown eight weeks before cuttings were taken. For the growth phase comparison study using LR64, four cuttings were taken for AP treatments for six weeks prior to inoculation and the JP treatment cuttings two weeks later four weeks prior to inoculation. One week following the last JP treatment cutting date, four cuttings were taken weekly for the race comparison study five weeks prior to inoculation. Eight cuttings of the LR67 F_2 plants were taken five weeks before inoculation with both races of the pathogen. Cuttings were rooted and planted into 50-cell tray as described in section 4.2.3. The photoperiod was changed to 18 hours after transplanting the cuttings to induce normal development through the physiological phases.

Table 6.1. Interspecific and Introgression Populations.

Population Name (Type)	Cross	Generation	Cotyledon Colour	% *Lens ervoides*	*Colletotrichum truncatum* Resistance Traits	Population Developed By
LR59 (Interspecific)	Eston / L-01-827A	$F_{7:9}$	Segregating for yellow and red	50%	Race Ct1 and Ct0	Fiala, 2006
LR64 (Introgression)	LR59-81 / CDC Redberry	F_2 and $F_{2:3}$	Segregating for yellow and red	25%	Race Ct1 and Ct0 Juvenile Phase Resistance	Vail, 2010
LR67 (Introgression)	LR59-81 / Eston	F_2 and $F_{2:3}$	Yellow	25%	Race Ct1 and Ct0	Vail, 2010

6.2.2 Experimental design, disease inoculation and disease rating

Experiments on the replicated F_2 plants were set up in a split-plot design where the main plot treatments were either a representative isolate of race Ct1 or race Ct0 or plants at different growth phases (juvenile versus adult) for race and growth phase comparison studies, respectively. Sub-plots were one cutting-derived plant of each F_2 individual, 15 cutting-derived plants of each parental line ('Eston', 'CDC Redberry' or LR59-81) and for the race comparison studies, one to two cutting-derived plants of an F_1 hybrid plant. Two blocks were inoculated and rated at one time which constituted an experimental repeat (Expt). Each experimental repeat was repeated four times for a total of eight replications.

Spore suspensions of *C. truncatum* were grown and harvested as described in section 4.2.2 and adjusted to 10^5 spores / mL. Inoculations were also performed as described in sections 4.2.2 and 4.2.3. In the misting chamber, trays inoculated with different races were physically separated from each other. Two weeks after inoculation, plants were rated for percentage of the main stem covered in lesions (SL), for percentage of the leave affected by disease (LL) and for the percentage of the shoots that had died due to disease (SDB) as described in section 4.2.2.

6.2.2.1 Race comparison study

For the race comparison studies, LR64 and LR67 populations were evaluated as separate studies with the same design in the spring of 2007 and in the fall of 2007, respectively. Plants had developed a mean of 10 nodes with two to three nodes that had flower buds or flowers five weeks after cuttings of F_2s were taken. Cutting-derived plants were then inoculated with 10 mL of spore suspension of either race Ct1 (isolate 95B36, ID#-35) or race Ct0 (isolate 95A8, ID#-34) of *C. truncatum* .

6.2.2.2 Phase comparison study

Prior to inoculation of the phase comparison study, the number of nodes and the number of nodes with flower buds were recorded for each plant. These data were analyzed as generalized linear models with normal distribution in the Genmod procedure in SAS. The JP plants had six to seven nodes of which most had no flower

buds. The AP plants had 11 to 12 nodes of which two to four nodes had buds or flowers. Contrast of the values between JP and AP plants for each line or the F_2s combined indicated highly significant differences (Appendix 6). Juvenile phased and AP plants were inoculated with approximately five or 10 mL, respectively, of spore suspension of race Ct0 isolate 95A8. Spore concentration and inoculation procedures were otherwise the same as in the race comparison study except that main plots were not physically separated.

6.2.2.3 $F_{2:3}$ families

For 99 $F_{2:3}$ families of the LR64 population, resistance to *C. truncatum* was tested in an outdoor disease nursery in 2008 at the North Seed Farm of the Department of Plant Sciences, University of Saskatchewan, Saskatoon. Eleven replicates of each of the parents were included as controls. In order to adjust for variability over the field, the 99 families plus the parental controls were arranged in 11 x 11 simple lattices using Square I, II and III as described in Cochran and Cox (1992) which constituted three replications (Rep) of the experiment. Each plot was flanked by plots of susceptible 'Eston'. Plots were approximately 75 cm long and had 20 seeds per plot planted with a row cone drill with 30 cm between rows at 2.5 cm depth. Emergence varied from two to 20 plants per plot. Approximately one month after planting, plots were inoculated with diseased straw collected from the previous year's anthracnose nursery. A misting irrigation system promoted disease development. Each square or replication was surrounded by four rows of barley to prolong canopy wetness throughout the day as irrigation occurred only in the evening, overnight and early morning. Approximately six weeks after inoculation, SLs were rated using the Horsfall Barratt scale as described in section 3.2.3.

Many of the F_2 individuals from the LR67 population did not produce sufficient seed for testing the complete population of the $F_{2:3}$ families under the same conditions as LR64. Therefore 48 $F_{2:3}$ families of LR67 were tested in a growth chamber experiment for segregation of resistance to race Ct0 of *C. truncatum* . The selected families represented a random sample of the F_2s based on disease scores. Two replications of each family were tested by randomly assigning two families to a

tray (Tray) with 50 cells. Twenty seeds of each family were planted alternately in the tray and the remaining 10 cells in the tray contained one of the parental lines or 'CDC Redberry'. Six weeks after planting, $F_{2:3}$ families were inoculated with race Ct0 using the protocol and isolate described above. Twelve trays were inoculated at once (Inoc) and the entire experiment was repeated once (Rep). Two weeks after inoculation, the SL, LL and SDB was assessed.

Using the same experimental design as used to test $F_{2:3}$ families of LR67, 48 randomly selected $F_{2:3}$ families of LR64 were tested for segregation of resistance in the JP to race Ct0 under controlled conditions. The plants were inoculated 23 days after seeding. Two replications of 20 plants from each family were tested as described in the race comparison study. Stem lesions, LL and SDB were also assessed two weeks after inoculation.

6.2.3 Statistical analysis

Levene's test for homogeneity of variance (Levene, 1960) indicated there were high levels of heterogeneity for residuals of experimental repeats, blocks, races and lines in all parameters in both populations when disease rating data for the F_2s in all experiments were analyzed. Various transformations (as described by Bowley, 1999) were tested in an attempt to correct the heterogeneity of errors, however heterogeneity was often irreconcilable. One or two sources of variation could be corrected across some parameters with different transformation methods and occasionally heterogeneity across lines could be adjusted by remodelling the mixed procedure statement so that lines were grouped individually for testing of significance.

Repeats of the experiments (Expt or Rep) were found to contribute very little to the overall variability in all experiments using covariance ratio testing in the mixed procedure, thus data were grouped together for further analysis (Table 6.8, Appendix 7 to 11). Least-square means of raw disease ratings were calculated for F_1, F_2s, parental and check lines in the Mixed procedure in SAS (SAS Institute, Cary, NC). Standard errors of differences and *p*-values for differences between pairs of means between race Ct1 and Ct0 were calculated on the parental checks, F_1 and pooled F_2s were calculated using the PDIFF option (Appendix 7 and 8). Similarly, individual F_2 means were

determined using the mixed procedure based on population data only (Table 6.8 and Appendix 9). Pearson correlations of F_2 individual means across the two races were calculated in SAS. Frequency distributions of the populations were determined and classification of plants being resistant or susceptible were performed as described in section 4.2.2. Using chi-square tests, seedling- and cutting-derived F_2s were tested for fit to Mendelian segregation ratios for resistance as described in section 4.2.2.

Individual F_3 plant ratings were classified into categories described above for frequency distribution analysis. Discrete classes were evident for studies performed using race Ct0 under controlled conditions. Means of the parents, check and pooled F_3s were determined in the Mixed procedure in SAS and individual $F_{2:3}$ family means were calculated based on population data only (Appendix 10 to 12). Plot or tray variance of the family was also subject to analysis in the mixed procedure to adjust for variability over the experiments.

Due to the continuous nature of the field-tested LR64 F_3 distribution and to support the hypothesis of major gene control in both populations for all traits, Fain's test (Lynch and Walsh, 1998) was performed on SL ratings. The test hypothesizes that if major gene(s) are involved in controlling the trait, $F_{2:3}$ families with the most extreme phenotypic means are most likely homozygous thus will have lower within family variance. Fit to the model is tested by regressing the family variance on the family mean where a significant quadratic term is an indication of a major gene or genes.

The $F_{2:3}$ family variance and the $F_{2:3}$ family means were also regressed on the individual F_2 means. $F_{2:3}$ families from LR67 were grouped into homozygous susceptible, segregating or homozygous resistant classes based on the comparison of F_2 means and $F_{2:3}$ family variances (Figure 6.1 and Appendix 13). Juvenile LR64 $F_{2:3}$ families were grouped into susceptible, segregating or resistant classes based on patterns of pair wise differences in variance of each of the families using the PDIFF option in the mixed procedure (data not shown). The resulting ratios were compared to classification of the F_2 individuals based on the mean and were subject to Chi-square analysis. For LR64, the ratio was tested for fit to the genetic model for dominant and recessive epistasis (7 resistant F_2 / resistant $F_{2:3}$: 6 resistant F_2 /

segregating $F_{2:3}$: 2 susceptible F_2 / segregating $F_{2:3}$: 1 susceptible F_2 / susceptible $F_{2:3}$) as well for duplicate and recessive epistasis plus a single recessive gene (21 resistant F_2 / resistant $F_{2:3}$: 22 resistant F_2 / segregating $F_{2:3}$: 14 susceptible F_2 / segregating $F_{2:3}$: 7 susceptible F_2 /susceptible $F_{2:3}$). For LR67, the expected $F_{2:3}$ ratio for duplicate recessive epitasis (1 resistant F_2 / resistant $F_{2:3}$: 8 resistant F_2 / segregating $F_{2:3}$: 7 F_2 /susceptible $F_{2:3}$) was tested.

6.3 Results

6.3.1 Major gene control of resistance traits

Frequency distributions of F_2 individuals suggested major gene control of resistance as bimodal distributions were observed in both populations on both resistance traits, with both races on the SL and SDB parameters (Table 6.2). This is consistent with the bimodal frequency distribution pattern observed on a larger sample of an additional 196 LR64 F_2s inoculated with a lower spore concentration of the race Ct0 isolate (Appendix 14). Discrete classes in the distribution of the LL were not as evident and the curve was skewed towards susceptibility. Higher levels of disease for this parameter were observed in the resistant parent LR59-81 (Tables 6.3 and 6.4) suggesting resistance expressed in the leaves may not be as highly expressed or controlled by the same major gene(s). On the other hand, this distribution could be partially due to the cutting method as a more continuous distribution was observed on LR64 F_2 cutting compared to seedlings in Chapter 4. Values for LR67 for all disease parameters appear to be skewed towards lower disease compared to both studies on LR64 (Table 6.2), consistent with overall disease observed in the LR67 F_2 experiments (Tables 6.3 and 6.4). Furthermore, bimodality on LR67 F_2s on SL means frequency distribution is less evident when infected with race Ct1 (Table 6.2).

The frequency distributions of F_3 values suggested non-normal distribution for both populations in all studies (Table 6.5) which was confirmed when the null hypothesis for normality was rejected (Appendix 15). Negative kurtosis values indicated platykurtic distributions (the peak was lower than expected for the normal) for field-tested LR64 and LR67 F_3s, but not for LR64 F_3s inoculated in the JP which

was leptokurtic. The LR64 population was positively skewed, towards the resistant parent, under both inoculation regimes while LR67 F_3s were negatively skewed towards the susceptible parent. Populations mean values were all significantly greater than the mid-parental values, however more so for the LR67 F_3s (Table 6.5 and Appendix 15). Distribution of the field-tested and JP (with controlled inoculations) LR64 F_3 SL and LL were relatively continuous with no clear classes distinguishing resistant and susceptible classes whereas bimodal distributions for LR67 F_3s and SDB of JP LR64 F_3s existed. Bimodality in LR67 F_3 compared to those of LR64 supports a simpler mode of inheritance in the susceptible background (Table 6.5). Fain's test supported control of resistance by major genes in both populations and under both inoculation regimes for LR64 as $F_{2:3}$ families with intermediate means had higher variances (Figure 6.1 and Appendix 13, 16 and 17). Quadratic terms were significant ($p < 0.05$ for LR64 $F_{2:3}$ families tested in the disease nursery and $p < 0.0001$ for LR64 and LR67 $F_{2:3}$ families tested under controlled conditions) when the regression analysis was performed.

Figure 6.1. Variance of stem lesions within a $F_{2:3}$ family as a quadratic function of the family mean when inoculated with *Colletotrichum truncatum* for LR67 and juvenile phased LR64 inoculated with race Ct0 and field-tested LR64.

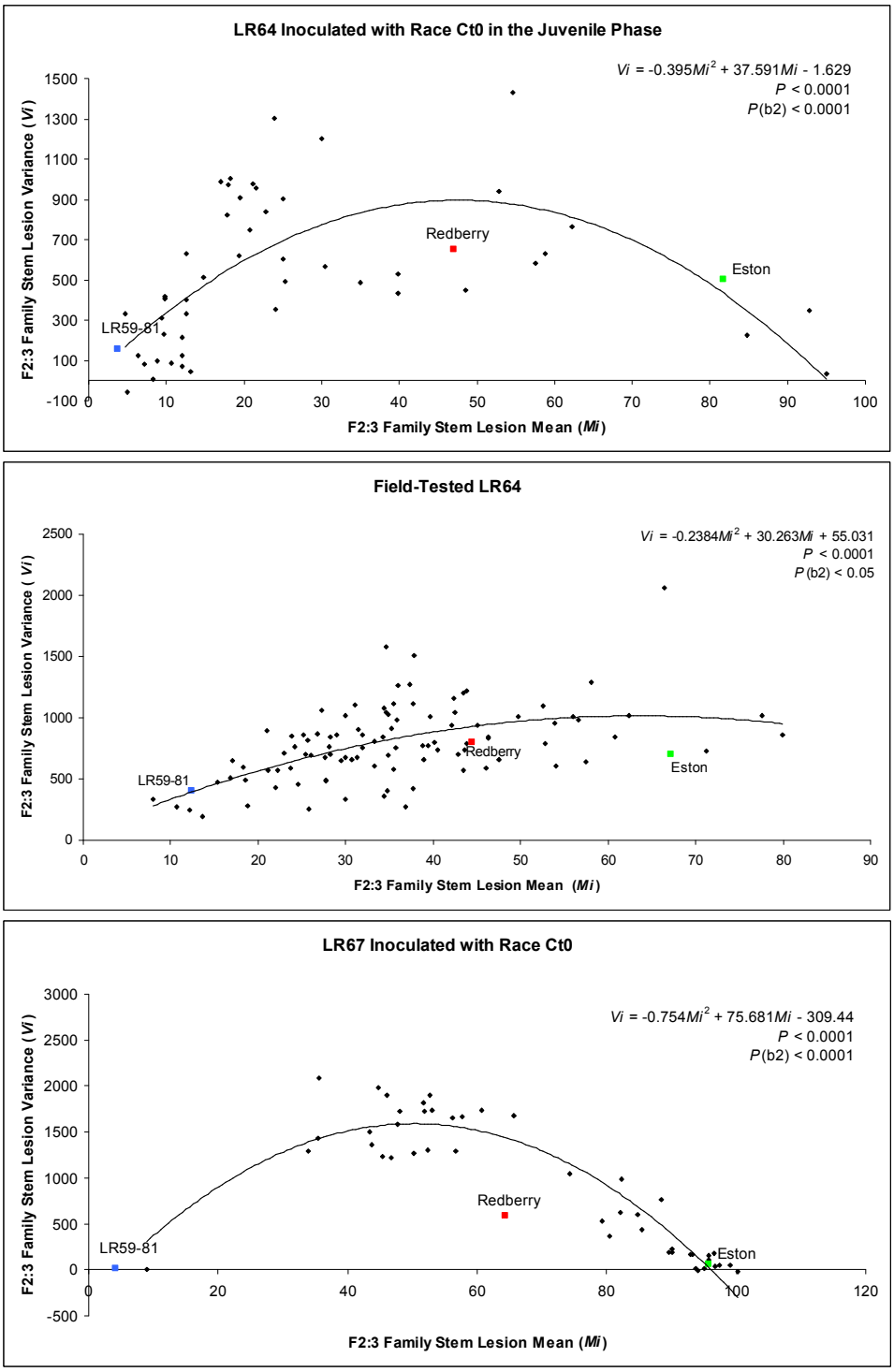


Table 6.2. Frequency distributions of stem and leaf lesions and shoot die back ratings of *Lens culinaris* F_2 individuals from LR 64 and LR67 populations inoculated in the adult phase with races Ct0 and Ct1 of *Colletotrichum truncatum* or race Ct0 at juvenile and adult phases.

	LR64						LR67						LR64			
	SL †		LL		SDB		SL		LL		SDB		SL		SDB	
Disease (%)	Ct1 ‡	Ct0 ‡	Ct1	Ct0	Ct1	Ct0	Ct1	Ct0	Ct1	Ct0	Ct1	Ct0	JP §	AP §	JP	AP
0	0	0	0	0	17	4	1	0	0	0	33	25	0	0	16	4
1-25	62	56	0	0	48	48	62	59	15	16	29	34	68	31	52	20
26-50	13	20	51	31	8	20	17	2	49	43	9	7	15	33	15	33
51-75	12	7	24	41	10	9	32	24	30	10	26	15	7	8	8	14
76-100	16	21	28	32	20	23	3	27	20	43	17	31	17	35	16	36
Total	103	104	103	104	103	104	115	112	114	112	114	112	107	107	107	107

† Percentage of the main stem covered in lesions (SL), leaf lesions (LL) and shoot die back (SDB).

‡ Race Ct1 and Race Ct0 of *Colletotrichum truncatum*.

§ Juvenile phase (JP) plants and adult phase (AP) plants.

Table 6.3. Mean stem and leaf lesions and shoot die back of parental and check lines, F_1 and pooled LR64 F_2 individuals inoculated in the adult phase with races Ct0 and Ct1 of *Colletotrichum truncatum* and significant differences of lines and race comparisons.

	Stem Lesions [†‡]			Leaf Lesions [†§]			Shoot Die Back [†¶]		
	Disease (%) [#]		Significance	Disease (%)		Significance	Disease (%)		Significance
Line	**Ct1**	**Ct0**	**Ct1vs.Ct0**	**Ct1**	**Ct0**	**Ct1vs.Ct0**	**Ct1**	**Ct0**	**Ct1vs.Ct0**
LR59-81	2.8	6.5	*	42.8	50.0	*	0.5	10.9	**
F_1	8.1	15.8	ns	46.2	49.0	ns	2.1	13.3	ns
F_2	31.5	36.0	**	59.2	63.9	ns	30.3	37.8	**
CDC Redberry	75.7	90.0	*	81.5	92.3	**	71.6	89.2	**
Eston	96.1	98.6	ns	97.8	98.3	ns	99.2	99.6	ns
				Significance					
LR59-81 vs. F_1	ns	ns		ns	ns		ns	ns	
LR59-81 vs. F_2	**	**		**	*		ns	ns	
LR59-81 vs. CDC Redberry	**	**		**	**		**	**	
LR59-81 vs. Eston	**	**		**	**		**	*	
F_1 vs. F_2	*	ns		ns	ns		**	**	
F_1 vs. CDC Redberry	**	**		**	**		**	**	
F_2 vs. CDC Redberry	**	**		**	**		**	**	
Eston vs. F_1	**	**		**	**		**	**	
Eston vs. F_2	**	**		**	**		**	**	
Eston vs. CDC Redberry	**	ns		**	ns		**	**	

† Significant differences in disease rating values between race Ct1 and race Ct0, * Significantly different from zero at $p < 0.05$; ** Significantly different from zero at $p < 0.01$.

‡ Significant differences tested based on values Cubic root transformed to adjust for heterogeneity of repeats and race.

§ Significant differences tested based on data grouped by line to adjust for heterogeneity.

¶ Significant differences based on the transformed square roots of values with $3/8^{th}$ added and grouped by line to adjust for heterogeneity of race and line.

Least Square Means determined in the mixed procedure using the repeated statement to group errors by line.

6.3.2 Genetic control of resistance to races of *Colletotricum truncatum*

The LR64 F_1 hybrid did not have significantly different disease levels than the resistant parent LR59-81 suggesting complete dominance of the trait (Table 6.3), whereas the LR67 F_1 hybrid had more disease than the resistant parent across all parameters assessed (Table 6.4). These differences were not large and ranged from approximately 2% to 16% suggesting very little dominance deviation. Conversely, the distributions of means of the F_2 versus $F_{2:3}$ families between the two populations suggest incomplete dominance of LR59-81 in LR64 and complete dominance in LR67 as LR67 data points are separated into two distinct clusters (Figure 6.2 and Appendix 17 and 18). It is possible that the field nursery conditions used for LR64 amplified the complexity of the genetic control of partial resistance under lower disease pressure. Furthermore, assessment of LR59-81 dominance in the LR64 population may be skewed by the presence of resistance genes from 'CDC Redberry'.

In all studies, significant differences for disease ratings were most consistently observed between the two races for 'CDC Redberry', supporting the differential ability of this line (Tables 6.3, 6.4 and 6.6). Differences between the two races were observed in the means of the F_2s for both SL and SDB when 'CDC Redberry' was the susceptible parent (LR64), whereas only SL differences between the two races were observed when 'Eston' was the susceptible parent (LR67) (Tables 6.3 and 6.4). Stem lesion and SDB means for F_2s were all skewed towards the resistant parent from the

mid-parental value for both populations. Leaf lesion means were skewed towards the susceptible parent 'CDC Redberry' across both races and was slightly skewed towards the susceptible parent 'Eston' when inoculated with race Ct0 (Tables 6.3 and 6.4).

When correlations of individual F_2 means for both races of *C. truncatum* were analyzed, the values were highly correlated and significant across all parameters assessed (Table 6.7) suggesting the same gene(s) are controlling resistance to both races or the genes are tightly linked on the same chromosome. This hypothesis was supported by non-significant Race x Line interaction in the analysis of variance across almost all parameters (Appendix 9).

Table 6.4. Mean stem and leaf lesions and shoot die back of parental and check lines, F_1 and pooled LR67 F_2 individuals inoculated in the adult phase with race Ct0 and race Ct1 of *Colletotrichum truncatum* and significant differences of lines and race comparisons.

	Stem Lesions [†‡]			Leaf Lesions [†§]			Shoot Die Back [†§]		
	Disease (%) [¶]		Significance	Disease (%)		Significance	Disease (%)		Significance
Line	**Ct1**	**Ct0**	**Ct1vs.Ct0**	**Ct1**	**Ct0**	**Ct1vs.Ct0**	**Ct1**	**Ct0**	**Ct1vs.Ct0**
LR59-81	3.4	2.6	ns	17.4	16.7	ns	2.3	0.2	ns
F_1	7.2	12.6	**	29.4	33.0	Ns	0.3	2.5	ns
F_2	29.1	39.5	**	48.5	54.5	ns	32.0	36.9	ns
CDC Redberry	42.4	58.2	*	49.8	64.7	*	21.5	44.9	ns
Eston	73.1	86.9	*	80.7	89.8	ns	79.0	87.1	ns
Significance									
LR59-81 vs. F_1	**	**		**	**		*	ns	
LR59-81 vs. F_2	**	**		**	**		**	**	
LR59-81 vs. CDC Redberry	**	**		**	**		**	**	
LR59-81 vs. Eston	**	**		**	**		**	**	
F_1 vs. F_2	**	**		ns	ns		ns	ns	
F_1 vs. CDC Redberry	**	**		*	ns		ns	**	
F_2 vs. CDC Redberry	**	**		ns	ns		*	*	
Eston vs. F_1	**	**		**	**		ns	**	
Eston vs. F_2	**	**		*	*		ns	*	
Eston vs. CDC Redberry	**	**		ns	ns		ns	ns	

† Significant differences in disease rating values between race Ct1 and race Ct0, * Significantly different from zero at $p < 0.05$; ** Significantly different from zero at $p < 0.01$.

‡ Significance of differences based on values Logarithmic transformed (base 100) and grouped by line to adjust for heterogeneity of race and lines.

§ Significance of differences based on data Reciprocal transformed and grouped by line to adjust for heterogeneity of race and line.

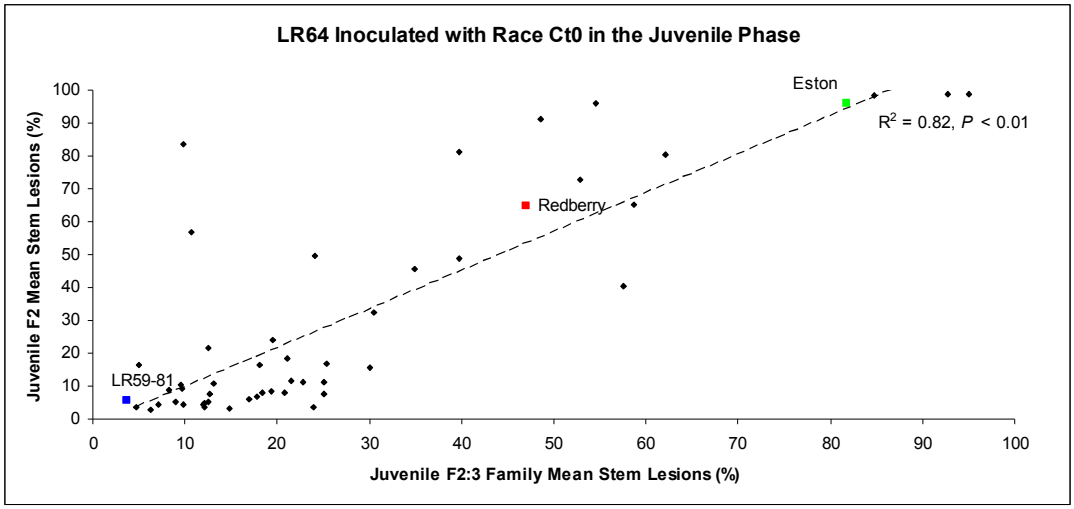
¶ Least Square Means determined in the mixed procedure using the repeated statement to group errors by line.

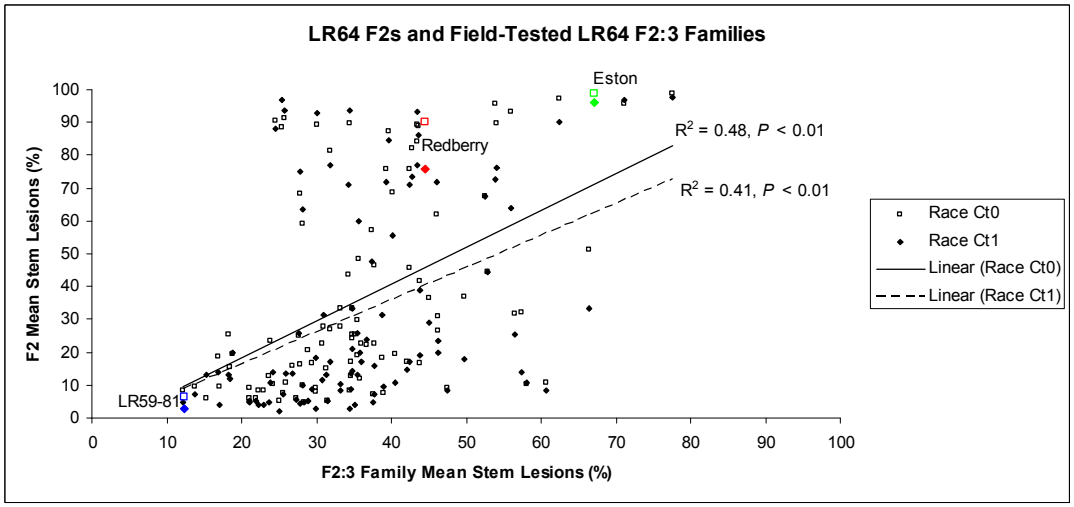
Table 6.5. Frequency distributions of stem and leaf lesions and shoot die back for F_3 individuals from LR64 from a 2008 disease nursery and juvenile-LR64 and adult-phased LR67 inoculated with race Ct0 of *Colletotrichum truncatum*.

		LR64								LR67 in the Adult Phase							
	Disease Nursery	Race Ct0 in Juvenile Phase								Race Ct0							
		SL				LL		SDB		SL		LL		SDB			
	SL†	n	%	n	%	n	%	n	%	n	%	n	%	n	%		
Disease (%)	0	0	0	0	0	0	0	1489	80	0	0	0	0	441	28		
	1-25	2242	58	1276	69	685	37	26	1	396	25	284	18	33	2		
	26-50	594	15	179	10	539	29	75	4	56	4	111	7	80	5		
	51-75	316	8	145	8	263	14	3	0	80	5	31	2	16	1		
	76-100	714	18	262	14	374	20	268	14	1037	66	1143	73	997	64		
	Total	3866		1862		1861		1861		1569		1569		1567			
Means (%)	All F_3 individuals	34.0		27.0		44.8		16.8		70.7		78.0		66.9			
	CDC Redberry	44.5		47.0		62.3		16.5		64.3		79.7		44.6			
	LR59-81	12.3		3.8		22.0		0.4		4.1		14.0		0.9			
	Eston	67.2		81.8		88.1		75.5		95.9		97.7		96.9			

† Percentage of the main stem covered in lesions (SL), leaf lesions (LL) and shoot die back (SDB).

Figure 6.2. Mean stem lesions of F_2 individuals inoculated with *Colletotrichum truncatum* versus $F_{2:3}$ family means for LR64 and LR67.





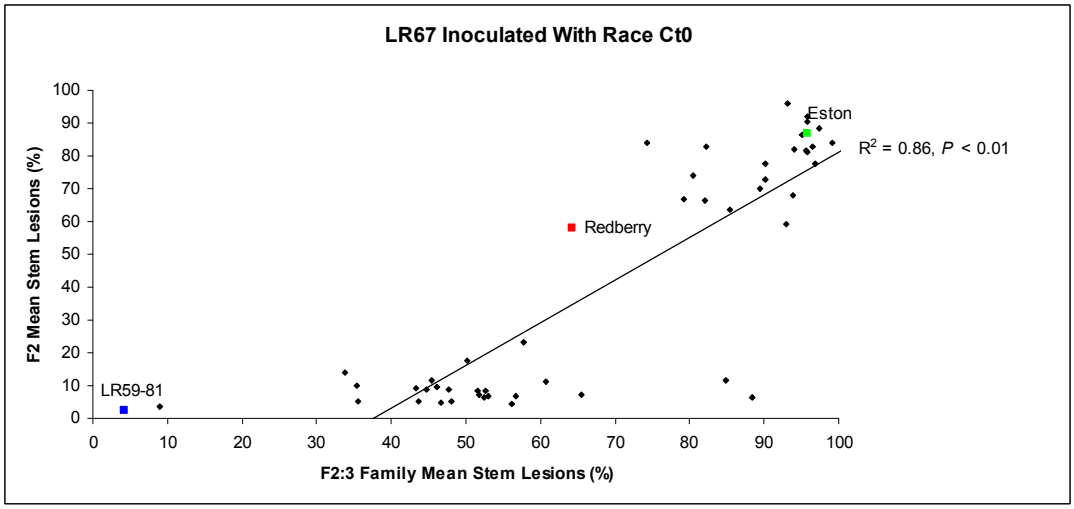


Table 6.6. Mean stem lesions and shoot die back of parental and check lines, pooled LR64 F_2 individuals inoculated with *Colletotrichum truncatum* race Ct0 at juvenile and adult phases and significant differences of lines and phase comparisons.

	Stem Lesions †‡			Shoot Die Back †		
	Disease (%) §		Significance	Disease (%)		Significance
Line	**JP**	**AP**	**JP vs. AP**	**JP**	**AP**	**JP vs. AP**
LR59-81	5.7	4.7	ns	6.3	11.3	Ns
F_2	28.9	51.4	**	28.1	54.2	**
Redberry	64.8	97.1	**	56.0	97.9	**
Eston	95.9	98.6	ns	94.9	100.0	Ns
			Significance			
LR59-81 vs. F_2	**	**		**	**	
LR59-81 vs. Redberry	**	**		**	**	
LR59-81 vs. Eston	**	**		**	**	
F_2 vs. Redberry	**	**		**	**	
Eston vs. F_2	**	**		**	**	
Eston vs. Redberry	**	ns		**	**	

† Significant differences in disease rating values between juvenile phase (JP) and adult phase (AP) plants, * Significantly different from zero at $p < 0.05$; ** Significantly different from zero at $p < 0.01$.

‡ Significant differences tested based on values Cubic transformed data grouped by line to adjust for heterogeneity of age and line.

§ Least Square Means determined in the mixed procedure using the repeated statement to group errors by age of plants.

Table 6.7. Correlations of ratings of stem and leaf lesion and shoot die back means of LR64 and LR67 F_2 individuals inoculated with races Ct0 and Ct1 of *Colletotrichum truncatum* or with race Ct0 at juvenile and adult phases.

	Race Ct1 vs. Race Ct0				Juvenile vs. Adult	
	LR 64		LR 67		LR 64	
	Correlation †	*p*-Value	Correlation	*p*-Value	Correlation	*p*-Value
SL ‡	0.94	<0.01	0.94	<0.01	0.88	<0.01
LL	0.67	<0.01	0.91	<0.01		
SDB	0.90	<0.01	0.94	<0.01	0.88	<0.01

† Pearson correlation coefficient

‡ Percentage of the main stem covered in lesions (SL), leaf lesions (LL) and shoot die back (SDB).

Several genetic models for one or two major genes were tested on the segregation ratios to determine how many genes might be controlling resistance to both races of the pathogen in LR59-81. The model for duplicate dominant epistasis (15 resistant : 1 susceptible) was consistently rejected with Chi-square testing across all parameters in both populations and for both resistance traits (data not shown). When ratios that were rejected or failed to be rejected were compared, there were several discrepancies between the two populations and between the races across the parameters (Table 6.9). When the resistant line LR59-81 was crossed with 'CDC Redberry' (LR64), there was consistent failure to reject the null hypothesis for a single dominant gene (3 resistant : 1 susceptible) with Chi-Square analysis for both SL and SDB parameters when inoculated with race Ct1 and race Ct0. However, when LR59-81 was crossed into a susceptible background with 'Eston' (LR67), this genetic model was rejected across all parameters over both races except for when inoculated with race Ct1 and evaluated based on SL. The most consistent ratio that failed to be rejected for the LR67 F_2s was that for duplicate recessive epitasis (9 resistant : 7 susceptible), especially for SDB. Genetic control by two genes with dominant and recessive epistasis (13 resistant : 3 susceptible) was consistently rejected in the LR67 F_2s, but was inconsistent in reactions on LR64 F_2s when inoculated with the two

different races of the pathogen. Based on these results, it was evident that genetic control of resistance contributed by LR59-81 appeared to be different in the different genetic backgrounds and for LR67, subject to the race of *C. truncatum* used despite the high correlation between the disease scores. Thus, the $F_{2:3}$ families were tested for resistance to confirm these results. Furthermore, the populations tested were not large enough to discriminate between segregation ratios tested based on the F_2 generation alone (Hanson, 1959).

Results on the $F_{2:3}$ families of LR67 supported the duplicate recessive epitasis model for genetic control observed in the F_2 generation of this population. Classification of $F_{2:3}$ families were compared to the classifications in the F_2 generation and the expected ratios were tested for fit to the genetic model for duplicate recessive epitasis. There was failure to reject the null hypothesis of this segregation pattern (Table 6.10). Comparisons of classifications between F_2 and $F_{2:3}$ showed that there were very few families (less than five) which fell outside the expected classifications between the two generations further supporting genetic control by this model (Table 6.10). Further supporting this genetic model was the comparison of the $F_{2:3}$ family variance with the F_2 mean, as well as the comparison of means between the two generations as patterns of plotted values followed the patterns expected for those genetic models (Figures 6.2 and 6.3; Appendix 18 and 19). Highly significant correlations between F_2s and $F_{2:3}$ family means existed for all parameters (Figure 6.2 and Appendix 18), presumably due to lack of representation of non- segregating resistant $F_{2:3}$ families.

6.3.3 Phase-dependant resistance in 'CDC Redberry'

Significant differences were consistently observed for both SL and SDB ratings between JP and AP plants for the mean of the F_2 individuals and on 'CDC Redberry' (Table 6.6). Despite highly significant correlations between JP and AP F_2 means (Table 6.7), the Phase x Line interaction was highly significant for SL percentage in the analysis of variance (Table 6.8) indicating differences in resistance between the two phases. When the significant pair-wise differences of F_2 individuals between the two phases were determined, it was found that 45% of the SL means and

36% of the SDB means were significantly different from each other (Table 6.11). Differences ranged from 23% to 51% for the SL and 30% to 63% difference for the SDB (data not shown). The percentage of F_2 individuals that switched classification from susceptible to resistant was 18% for the SL and 20% for the SDB (Table 6.11).

Table 6.8. Mixed model analysis of variance of stem lesion and shoot die back percentage on *Lens culinaris* F_2 individuals from LR64 inoculated with *Colletotrichum truncatum* race Ct0 at juvenile and adult phases.

Source of Variation	Num df	Stem Lesions (%)				Shoot Die Back (%) †			
		Den df	F-Value	*p-Value*	Estimate	Den df	F-Value	*p-Value*	Estimate
Phase	1	11.7	78.74	<0.01		3.07	57.60	<0.01	
Line	106	304	22.85	<0.01		306	13.94	<0.01	
Phase x Line	106	304	2.20	<0.01		305	1.20	0.12	
Expt					40.49				0.4457
Expt (Bloc)					0.00				0.0000
Expt x Phase					0.00				0.0031
Expt (Bloc x Phase)					19.42				0.0726
Expt x Line					29.03				0.0140
Expt x Phase x Line					28.23				0.2219
Residual					482.55				1.6001

† Values cubic transformed to adjust heterogeneity of Expt and Phase.

When the resulting ratios of resistant to susceptible F_2 LR64 plants, inoculated in the different phases, were subject to Chi-square analysis for various genetic models, it was found that different models fit the JP versus AP data (Table 6.9). At the JP, duplicate recessive epitasis (9 resistant : 7 susceptible) was rejected for both SL and SDB parameters whereas single dominant gene (3 resistant : 1 susceptible) and dominant and recessive epistatic (13 resistant : 3 susceptible) models were not rejected suggesting one of these two models reflect genetic control of the JP resistance trait. This was relatively consistent with the results from the same LR64 F_2 individuals when inoculated with both races of *C. truncatum*. When inoculated while F_2s were in the AP, both single dominant gene and dominant and recessive epistasis models were rejected and the model for duplicate recessive epitasis (9 resistant : 7 susceptible) was not rejected for both parameters. The results for AP F_2s were very similar to the

results for the LR67 F_2s especially when inoculated with race Ct0. This suggests the differences in maturity in the LR64 AP cuttings and inoculation with the more virulent race of the pathogen on LR67 removed the convoluting effect of the phase-dependent resistance found in 'CDC Redberry' and the effect of minor resistance genes that may have provided some limited protection from race Ct0 in the LR64 population.

When the differences between the number of genes controlling resistance between the two phases was considered, it was hypothesized a single recessive gene may be responsible for partial resistance in 'CDC Redberry' in the JP. Buchwaldt et al. (2001) reported that resistance to race Ct1 in 'Indianhead' is controlled by a single recessive gene and this line is considered the source of resistance found in 'CDC Redberry' (Vandenberg et al., 2006). Thus, ratios for duplicate recessive epistasis along with a single recessive gene were tested on all LR64 results. There was failure to reject this model for LR64 F_2s inoculated with the two different races of the pathogen as well as in the JP inoculated with race Ct0. Results were inconclusive in the AP when both SL and SDB were considered (Table 6.9). This proposed model was the best fit with all parameters on the larger population of F_2s screened with a lower spore concentration of race Ct0 inoculated when the plants were just beginning to flower (Appendix 20).

Table 6.9. Chi-Square test of genetic models on resistant and susceptible F_2 individuals from LR67 and LR64 inoculated in the adult phase with race Ct0 and race Ct1 of *Colletotrichum truncatum* and LR64 inoculate with race Ct0 at juvenile and adult phases for stem lesions and shoot die back.

	LR64 in Adult Phase				LR67 in Adult Phase				LR64 inoculated with Race Ct0					
		Race Ct1 [†]		Race Ct0		Race Ct1		Race Ct0		JP [†]		AP		
	N [‡]	103		104		114		112		107		107		
	Ratio [§]	75:28		76:28		80:35		61:51		83:24		64:43		
	Genetic Model	χ^2 [¶]	*p*-Value [#]	χ^2	*p*-Value	χ^2	*p*-Value	χ^2	*p*-Value	χ^2 [a]	*P* [s]	χ^2	*p*-Value	
Stem Lesions	3:1	0.18	0.67	0.14	0.71	2.96	0.09	26.95	<0.01	0.28	0.59	12.56	<0.01	
	13:3	4.12	0.04	3.93	0.05	11.6	<0.01	54.76	<0.01	0.69	0.41	30.37	<0.01	
	9:7	11.06	<0.01	11.64	<0.01	10.04	<0.01	5.85	0.02	19.35	<0.01	0.49	0.49	
	43:21	0.36	0.24	1.50	0.22					4.99	0.03	2.22	0.14	
	Ratio	73:30		72:32		71:43		66:46		83:24		57:50		
	Genetic Model	χ^2	*p*-Value	χ^2	*p*-Value	χ^2	*p*-Value	χ^2	*p*-Value	χ^2	*p*-Value	χ^2	*p*-Value	
Shoot Die Back	3:1	0.77	0.38	1.63	0.20	11.29	<0.01	16.63	<0.01	0.28	0.59	26.08	<0.01	
	13:3	6.4	0.01	8.90	<0.01	28.72	<0.01	37.95	<0.01	0.69	0.41	52.36	<0.01	
	9:7	8.59	<0.01	6.87	<0.01	2.67	0.10	0.89	0.35	19.35	<0.01	0.22	0.64	
	43:21	0.57	0.45	0.16	0.69					4.99	0.03	8.58	<0.01	

† Race of *Colletotrichum truncatum* or phase inoculated at with race Ct0 of *C. truncatum* or juvenile phase (JP) or adult phase (AP) of plants when inoculated with race Ct0

‡ Number of F_2 individuals analyzed

§ Resistant : Susceptible with resistant individuals having ≤50% disease based on the least square mean

¶ X^2 value for test of given ratio

Probability of a greater χ^2 value under the null hypothesis of ratio tested with Yates correction for continuity

Table 6.10. Comparison of disease resistance classifications of F_2 individuals and $F_{2:3}$ families from LR64 inoculated at the juvenile phase and adult-phased LR67 with *Colletotrichum truncatum* race Ct0 for stem and leaf lesions and the shoot die back.

		LR64		LR67		
F_2 Class †	**$F_{2:3}$ Class** ‡	**SL (n)** §	**SDB (n)**	**SL (n)**	**LL (n)**	**SDB (n)**
Resistant	Resistant	24	24	1	1	1
Resistant	Segregating	12	13	22	22	22
Resistant	Susceptible	1	0	2	2	4
Susceptible	Resistant	4	3	0	0	0
Susceptible	Segregating	3	5	0	0	1
Susceptible	Susceptible	4	3	23	23	20
Total						
Ratio		24:12:3:4 ¶	24:13:5:3 ¶	1:22:23 #	1:22:23 #	1:22:20 #

	χ^2 ††	*p*-Value ‡‡	X^2	*p*-Value	χ^2 §§	*p*-Value	χ^2	*p*-Value	χ^2	*p*-Value
Chi-Square	4.20	0.24	1.94	0.59	1.66	0.44	1.66	0.44	1.04	0.59
	χ^2 ¶¶	*p*-Value	X^2	*p*-Value						
	11.04	0.01	10.79	0.01						

† Classification of F_2 individual where resistant had ≤50% disease

‡ Classification of $F_{2:3}$ family inoculated at the juvenile phase based on clusters present in pair wise comparisons of family variances for LR64 or classification of $F_{2:3}$ family based on clusters present in the regression of F_2 means on $F_{2:3}$ family variance for LR67

§ The number of F_2 individuals and $F_{2:3}$ families classified accordingly based on percentage of the main stem covered in lesions (SL), leaf lesions (LL) or shoot die back (SDB).

¶ F_2 Resistant / $F_{2:3}$ Family Resistant : F_2 Resistant / $F_{2:3}$ Family Segregating : F_2 Susceptible / $F_{2:3}$ Family Segregating : F_2 Susceptible / $F_{2:3}$ Family Susceptible with resistant plants having ≤50% disease

Note: The five LR64 F_2 / $F_{2:3}$ lines not conforming to classifications described were removed from the analysis.

F_2 Resistant / $F_{2:3}$ Family Resistant : F_2 Resistant / $F_{2:3}$ Family Segregating : F_2 Susceptible / $F_{2:3}$ Family Susceptible with resistant plants having ≤50% disease

Note: The two (SL & LL) or five (SDB) LR67 F_2 / $F_{2:3}$ lines not conforming to classifications described were removed from the analysis.

†† X^2 value for test of 7:6:2:1 ratio

‡‡ Probability of a greater χ^2 value under the null hypothesis of tested ratio

§§ X^2 value for test of 1:8:7 ratio

¶¶ X^2 value for test of 21:22:14:7 ratio

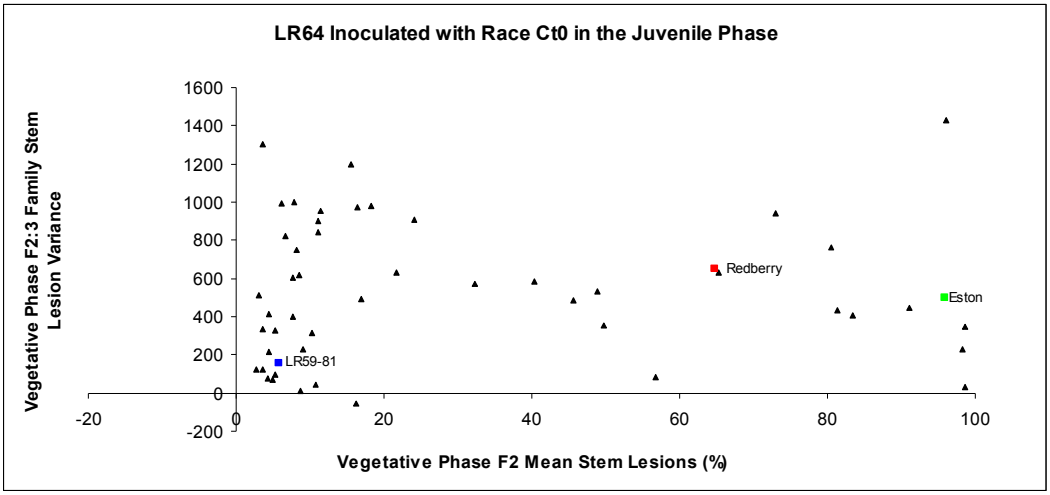
Table 6.11. Significant differences between means of juvenile and adult phases on F_2 individuals from LR64 inoculated with *Colletotrichum truncatum* race Ct0 for stem lesions and shoot die back.

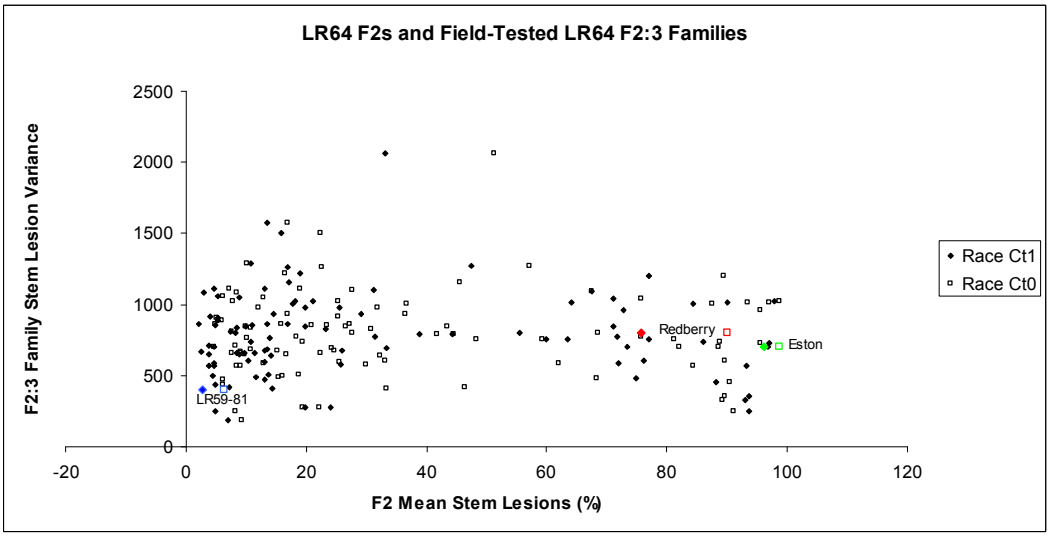
Juvenile Class	Adult Class	Stem Lesions		Shoot Die Back	
		n †	% of Total ‡	n	% of Total
Susceptible	Susceptible	6	6	11	10
Resistant	Susceptible	19	18	22	20
Resistant	Resistant	24	22	6	6
Total		49		39	

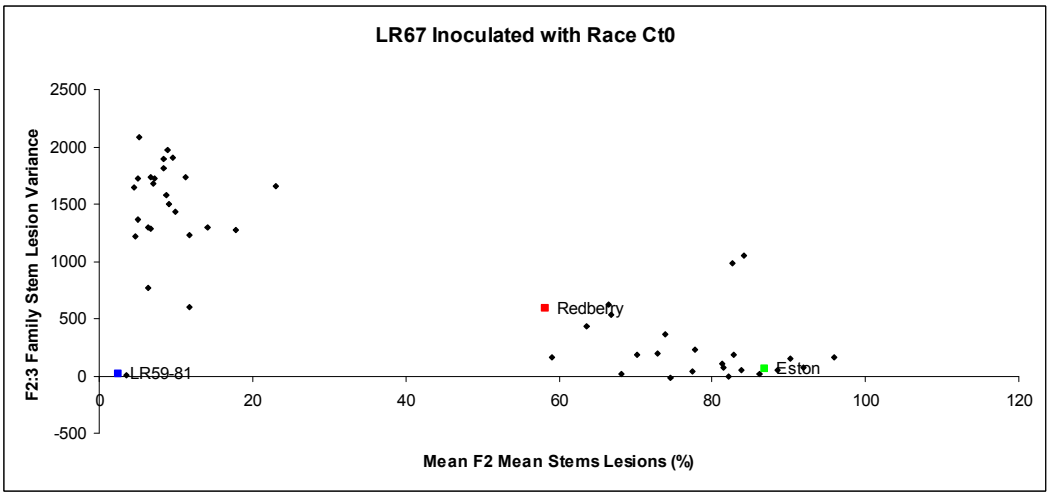
† Number of F_2 individuals showing significant differences between juvenile phase mean and adult phase mean ($p < 0.05$)

‡ The number of F_2 individuals as a percentage of the total number of F_2s

Figure 6.3. Mean stem lesion percentage of F_2 individuals inoculated with *Colletotrichum truncatum* versus $F_{2:3}$ family variance for LR64 and LR67.







When the classifications of LR64 F_2 individuals and $F_{2:3}$ families inoculated in the JP with race Ct0 of *C. truncatum* were analyzed, the dominant and recessive epistatic model was not rejected for both disease parameters (Table 6.10) whereas the single dominant gene model was rejected (data not shown). This supported the hypothesis that genetic control during the JP is due to two genes, one dominant and the other recessive. The hypothesis of duplicate recessive epistasis plus a single recessive gene was not rejected, but was much less convincing than the model for dominant and recessive epistasis when the $F_{2:3}$ family classifications were considered (Table 6.10). When the significant pair wise differences were considered, less than the expected one quarter (18% and 20% for SL and SDB, respectively) changed disease resistance classifications. These results suggest the contribution to resistance from 'CDC Redberry' may be due to minor genes that cannot be detected using qualitative analysis. Significant correlations between F_2s and $F_{2:3}$ family means existed for LR64 at both the JP as well as between field tested $F_{2:3}$ families and F_2s inoculated with both races of *C. truncatum* (Figure 6.2 and Appendix 17). Distributions of these comparisons support the dominant and recessive epistatic model with the majority of the families being either resistant in the F_2 and $F_{2:3}$ generations or resistant in the F_2 and segregating $F_{2:3}$ families, similar to the comparison of the F_2 individual means with the $F_{2:3}$ family variance (Figure 6.3 and Appendix 21).

The results of the investigation of genetic control of resistance in the AP plants seem to be contradictory when the same populations were inoculated with two different races of the pathogen. A plausible explanation is that there were higher disease levels in the AP plants than in the race-comparison experiment. The disease levels on 'CDC Redberry' and the F_2 mean in AP are approximately 7% and 15% higher, respectively, than race Ct0-inoculated plants (Tables 6.3 and 6.6). This is most likely due to the AP plants being inoculated six weeks after cuttings were taken rather than at five weeks after cuttings were taken in the race-comparison experiment. Furthermore, the environmental conditions in the phase-comparison experiment may have been more conducive to better differentiation by minimizing the effect of partial resistance found in 'CDC Redberry', although attempts were made to replicate conditions across both experiments. The results on the genetic control experiments for

resistance in LR64 could be skewed by the presence of minor resistance genes. Presumably, the effects of minor resistance genes were removed in LR67 when inoculated with race Ct0 as this population was created with the susceptible parent 'Eston'. This could be further examined by inoculating AP $F_{2:3}$ families under controlled conditions to better differentiate segregating families in the LR64 population, seed quantity permitting.

6.3 Discussion and Conclusions

Based on the results from both populations, it is clear that resistance in LR59-81 is controlled by major genes. This is supported by bimodal frequency distributions of the F_2 individuals and regressions of $F_{2:3}$ family variances on F_2 and $F_{2:3}$ family means. Results were relatively consistent across the three parameters measured, with the exception of the percentage of leaves affected by disease which showed a continuous distribution skewed towards susceptibility in the F_2 generation. Based on the results, it is clear that resistance in LR59-81 is dominant, however whether it is completely dominant or partially or incompletely dominant remains unclear. Genetic control of resistance in LR59-81 to both races of *C. truncatum* appears to be due to either the same gene, or tightly linked genes based on the correlation results. Tight linkage of genes or single gene control of both races of *C. truncatum* observed in these studies was also observed by Fiala et al. (2009) who found that in the primary interspecific population, very few disease class differences were observed between RILs inoculated with the two races. Linkage blocks are commonly observed when introgressing genes from wild relatives (Young and Tanksley, 1989), thus it is difficult to assess if both races are controlled by the same gene(s) or linked genes. A broad-spectrum resistance gene to three races of *Colletotrichum trifolii* was found in *Medicago truncatula* and transformed successfully to alfalfa (*M. sativa*) where only partial resistance was found (Yang et al., 2008a). It was suggested the *M. truncatula* resistance gene may be evolving slower than genes found in *M. sativa* as homologs of the gene were found to confer partial resistance, possibly related to the tetraploidization of *M. sativa*. Resistance to two different races of *Puccinia striiformis* F.sp. *tritici*, causing stripe rust in wheat, were found to be conferred by a single

dominant gene, however the two races were weakly virulent compared with other races in the pathosystem (Lin and Chen, 2007). To determine if the same gene is controlling resistance to the different races, one would need to examine genetic control in the resistant *L. ervoides* parent in a susceptible *L. ervoides* background. However, identifying a highly susceptible *L. ervoides* parent has been problematic because high levels of susceptibility are rare and when found, do not seem to easily hybridize with L-01-827A (data not shown).

Both F_2 and $F_{2:3}$ family results supported different models for genetic control of resistance for the two populations. When the resistant LR59-81 was crossed with 'CDC Redberry' (LR64), genetic control of resistance to both races appeared to be due dominant and recessive epistasis. However, when crossed with a susceptible parent 'Eston' (LR67), genetic control appeared to be due to duplicate recessive epistasis. When tested with the more virulent race Ct0 at latest maturity, the LR64 population was consistent with the duplicate recessive epistasis model, presumably since the JP-associated resistance effects from 'CDC Redberry' were removed. This is consistent with the results from Chapter 4 on the more mature cutting-derived LR64 F_2s that were compared to the seedlings (Table 4.5). Very few examples of resistance being controlled by duplicate recessive epistasis exist in published literature. When ascochyta resistance was transferred from *L. ervoides* and *L. orientalis* into *L. culinaris*, it was found that the interspecific F_1 indicated dominance of resistance and the F_2 segregation ratio was 9 resistant : 7 susceptible suggesting similar genetic control of resistance as was observed in these studies. However, these segregation ratios were found from crosses of susceptible parents (Ahmad et al., 1997). In cotton, resistance to leaf curl had a similar epistatic model, but the populations where it existed accounted for a low proportion of the total number of crosses examined (Lal Ahuja et al., 2007). Resistance to *Xanthomonas axonopodis* pv. *dieffenbachiae* in *Anthurium andraeanum* was found to be due to duplicate recessive epistasis where the presence of the either homozygous recessive gene conferred resistance (Elibox and Umaharan, 2008). Given the dominance demonstrated in the LR67 F_1 and the comparison of the means of F_2 versus $F_{2:3}$ families, it is more likely susceptibility is conferred by one of two homozygous recessive genes. Biologically it is hard to

rationalize how this could be. It is hypothesized that instead of susceptibility in the populations being conferred by two recessive genes, that this segregation ratio actually reflects an inability to maintain homozygous alien alleles from *L. ervoides*. Linkage maps of LR59, LR64, LR67 and *L. ervoides* are currently being developed using cross-legume conserved orthologous sequence markers and it is expected the resulting maps could be used to test the hypothesis that the lack of true breeding resistant families in both introgression populations is due to the resistance gene(s) being maintained in an interspecific heterozygote state. Furthermore, tracking the portions of the genome transferred by linkage mapping of the populations and comparing marker data with disease scores will help differentiate these seemingly conflicting results between studies.

Segregation distortion is commonly observed in interspecific crosses between plant species (eg. Patterson et al., 1988; Tadmor et al., 1987; Yang et al., 2008b; Zamir and Tadmor, 1986). It was expected that disease rating distributions from the raw RIL populations from which LR59-81 was selected were distorted from plausible Mendelian segregation, possibly due to incompatibilities between the genomes of the species (Fiala et al., 2009). The results from the current studies suggested that when the RIL was introgressed into adapted background, distortion may have still occurred. It is possible that distortion could have occurred as early as the formation of the F_2 population from the hybrid because the populations are highly skewed towards susceptible families. This suggests that one or two alleles derived from the resistant wild parent may contribute to meiotic instability. This was found in an interspecific population of cultivated backcross *Lycospersicon* plants where 21 regions across the linkage map were found to be distorted, of which 12 regions were heterozygous for the wild and cultivated alleles and nine were homozygous for the cultivated parental alleles (Patterson et al., 1988). It is also possible the results are due to aneuploidy created during the interspecific hybridization process possibly inadvertently creating alien addition lines. If the observed results are a result of inability to support homozygous alleles derived from *L. ervoides*, then the results provide insight into the populations sizes needed to introgress *L. ervoides* into the cultivated background. For example, it appears as though only one in 48 of the LR67 families was true breeding

for resistance, thus substantial population sizes would be needed within the breeding program to find individuals homozygous for the trait in combination with all other traits.

In interspecific populations between *L. ervoides* and *L. culinaris*, segregation distortion has been reported previously by Zamir and Tadmor (1986) who observed this in 44% of loci examined. They did not, however, find skewing of favoured alleles towards either species. Tadmor et al. (1987) found with simple linkage maps that single loci were independently distorted in an interspecific *Lens* cross suggesting only portions of, not entire chromosomes, are distorted. Linkage mapping of interspecific populations in both *Lycospersicon* and *Glycine* have been useful in identifying regions of the genome with distorted segregation (Patterson et al., 1988 and Yang et al., 2008b). Comparing interspecific (LR59) and introgression (LR64 and LR67) *Lens* maps with a non-distorted map in the wild *L. ervoides* species would help identify which regions of the alien chromosomes may contribute to meiotic instability and sterility during introgression. It would also resolve whether or not one or two loci on the map are actually involved in controlling resistance. If there are regions that contribute to meiotic instability, they could be selected against when backcrossing genes of interest into the adapted background. Furthermore, if traits of interest in *L. ervoides* occur in these regions, suitable maps would be useful in determining if introgression should be attempted.

Cytogenetic analysis of the introgressing chromosome pieces would help resolve the actual biological mechanisms that contributed to these results. For example, abnormal chromosome rearrangements were observed during meiosis in *L. culinaris* x *L. lamottei* hybrids with extra pieces of a chromosome in the tetrad formation of microspores and bridges where chromosomes had not separated properly (Fiala, 2006). Ladizinsky et al. (1985) observed different chromosomal pairing patterns in three different *L. ervoides* x *L. culinaris* crosses, two of which had several chromosomal rearrangements that resulted in no viable seeds. The other hybrid had a single quadrivalent in MI of meiosis and produced viable seeds, but had 46% pod abortion and only 52% pollen fertility. Segregation of F_2s from the hybrid for the quadrivalent fit the expected ratio for segregation of a reciprocal translocation.

Tadmor et al. (1987) found that when pollen viability of interspecific F_2s between *L. culinaris* and *L. ervoides* was good (greater than 85%), cells with normal bivalents were observed. If pollen viability was low (less than 65%), five bivalents and one quadrivalent were observed in diakinesis. This suggested that low pollen quality was associated with heterozygosity for a reciprocal translocation that differentiates the two species. However, Ladizinsky et al. (1985) did not observe any consistency between the pattern of chromosome associations and pollen stainability. Pollen quality of LR59-81 has not been assessed but it may be low because of low success of reciprocal crosses where LR59-81 was the pollen donor. While attempting the reciprocal crosses, it was visually observed that a very low proportion of LR59-81 flowers had healthy, viable pollen. It is possible that low pollen quality in LR59-81 could be also associated with the quadrivalent that is due to an ancient translocation between the species (Tadmor et al., 1987). Ladizinsky et al. (1985) described interspecific hybrids with pods that developed for seven to14 days after anthesis and then collapse and develop shrivelled non-viable seed. This was also observed in the LR59-81 parent and in its hybrids with *L. culinaris* lines. The breakdown of interspecific hybrid embryos could be caused by malfunction of the endosperm or interaction between the maternal cytoplasm and nuclear genomes (Ladizinsky et al., 1985).

In earlier studies examining the effect of growth phase and plant age on resistance to *C. truncatum* race Ct0, it was found that 'CDC Redberry' had higher levels of resistance in the JP than in the AP and that resistance seemed to be acquired as the plant progressed through the JP. This pattern of resistance decrease on 'CDC Redberry' was consistent when inoculated with race Ct1 of *C. truncatum* (Chapter 5). Results of the studies presented above showed that juvenile resistance in 'CDC Redberry' appears to be oligogenic with decreasing effects as the plant progresses through the AP. Similarly, quantitative trait loci analysis have shown different regions of the genome controlling resistance at different phases in resistance to *Stagonospora nodorum* in wheat (Shankar et al., 2008) and to turnip mosaic virus in Chinese cabbage (Zhang et al., 2008). Furthermore, Bilgic et al. (2006) found resistance to a single pathotype of *Cochliobolus sativus*, causing spot blotch in barley, was conferred by two genes at the seedling stage, but only one gene at the adult stage.

Differences in maturity in segregating lentil populations and the age at which plants are inoculated may both be considerations in outdoor disease nurseries and indoor screening for anthracnose resistance in the lentil breeding program at the Crop Development Centre. Depending on the season and timing of inoculation, the onset of disease epidemics in field nurseries may often occur when the plants are still in the JP, thus AP susceptibility is not selected against. Furthermore, in the case of populations derived from crossing genetically divergent material, pleiotropic effects of flowering date and maturity may convolute selection for true resistance. The timing of heading date when selecting for *Stagonospora nodorum* resistance in wheat has had similar challenges (Shankar et al., 2008). Typically, indoor inoculations of segregating lentil populations with *C. truncatum* are done four weeks after planting before the flowering stage. Results from this study suggest that in populations where 'CDC Redberry' has been used, adult resistance should be selected for when the plants are at least in the mid-flower stage. Comparisons of the results from the race and phase-comparison studies suggest the later the plants are screened, the more likely juvenile resistance will not be a factor.

Overall, it can be concluded that introgression of resistance genes to *C. truncatum* from *L. ervoides* accession L-01-827A is possible and will provide unprecedented levels of resistance in lentil to both races of the pathogen. However, based on these results, it is evident that a substantial number of strategic backcrosses into the adapted *L. culinaris* parent are required as it is possible that fertility problems associated with the wild gametes may persist. Larger populations sizes may be needed to allow for selection of favourable recombination where chromosomal rearrangement and sterility can be selected against. Furthermore, multi-generation testing may also be necessary for selecting resistance in order to differentiate true-breeding and segregating families. When the appropriate molecular tools are available for lentil, marker-assisted backcrossing and marker-assisted selection for major resistance genes could be efficient tools to quickly integrate the resistance gene(s) into new lines.

6.4 Prologue to Chapter 7

The interspecific RIL used as a parent in both LR64 and LR67 populations showed signs instability as at the F_{10} generation, seed derived from a single red cotyledon LR59-81 plant still segregated for approximately 85% red and 15% yellow cotyledon colour. The yellow cotyledon seeds produced chlorophyll deficient, infertile, non-viable mutant plants (data not shown). It was expected LR64 lines would all have red cotyledons based on red cotyledons in both parents (LR59-81 and 'CDC Redberry'). However, there was segregation for both cotyledon colour and chlorophyll-deficient mutants in the F_2 and $F_{2:3}$ generations of LR64 (Table 6.1). Interestingly, LR67 F_2 and $F_{2:3}$ generations were not only more uniform with normal chlorophyll production compared to F_2 and $F_{2:3}$ generations of LR64, but were also true breeding for cotyledon colour (all yellow) suggesting the allele for cotyledon colour of the LR59-81 female gamete in the creation of LR67 was yellow and that repeated backcrossing to 'Eston' may have reduced variability for plant type in the segregating population.

The genetics controlling cotyledon colour in lentil had previously been extensively studied (Slinkard, 1978; Vandenberg and Slinkard, 1987). Furthermore, segregation in *Lens* populations for albino and chlorophyll-deficient mutants had previously been described (Ladizinsky et al., 1985; Vandenberg and Slinkard, 1987; Vandenberg and Slinkard, 1989). It was proposed the segregation for these traits in the LR64 population could be used to gain insight on the instability exhibited by LR59-81 for the traits by comparing results to previous reports. The hypothesis is that segregation for cotyledon colour and albino traits will support the postulation that segregation for disease resistance in LR64 and LR67 populations are skewed.

CHAPTER 7

SEGREGATION OF COTYLEDON COLOUR AND CHLOROPHYLL DEFICIENT MUTANTS IN THE POPULATION LR59-81 X 'CDC REDBERRY'

7.1 Introduction and Objectives

Vandenberg and Slinkard (1987 and 1989) described two types of chlorophyll deficient mutants, *xantha* and *chlorina*. The *xantha* mutant was a chlorophyll-deficient mutant which emerged with bright yellow tissue and died after three to four weeks. These mutants developed only from seed with yellow cotyledons and were caused by a single homozygous recessive allele that blocks normal cholorophyll production that also blocks production of red or green cotyledon colour. The population exhibiting *xantha* seedlings was originally described as being derived from a hybrid between lentil subspecies *L. culinaris* ssp. *culinaris* and *L. culinaris* ssp. *orientalis* (Vandenberg and Slinkard, 1987). *Chlorina* chlorophyll deficient mutants emerged with pale yellowish-green foliage and died within three weeks. The first two leaves of seedlings were normal in size, but subsequent leaves were progressively smaller. These mutants were found to spontaneously occur in a *L. culinaris* landrace. A single recessive gene is responsible for the *chlorina* mutant and epistatic interactions with cotyledon colour genetics are not exhibited by these mutants as in the *xantha* mutants (Vandenberg and Slinkard, 1989).

Ladizinsky et al. (1985) described segregation for unviable albino plants in an interspecific F_2 population between *L. culinaris* and *L. ervoides* and suggested the phenotype was conferred by two recessive genes. It was suggested that this was due to a single gene for chlorophyll production in each species that is not located on the same chromosome due to a minor translocation. Red cotyledon colour has previously

been shown, in two *L. culinaris* accessions, to be conferred by a single dominant allele and over alleles associated with yellow and green cotyledons (Slinkard, 1978).

The population LR64 (LR59-81 x 'CDC Redberry'), was segregating for cotyledon colour (Table 6.1) and albino or chlorophyll-deficient mutants, *xantha*-type mutants. It was hypothesized that by examining traits where genetic control has been previously elucidated, insight into the results on the disease resistance traits might be obtained. It was hypothesized that segregation for cotylelon colour in the LR64 population would depart from that expected for a single dominant gene and that segregation of chlorophyll-deficient mutants would resemble that observed previously in interspecific populations.

7.2 Materials and Methods

When LR64 F_2 plants were grown, individual plant phenotypes were recorded as having normal chlorophyll production or as chlorophyll-deficient mutants. When 48 LR64 $F_{2:3}$ families were grown for indoor disease inoculation, the number of chlorophyll-deficient mutants in each of the families were recorded. Of the 29 segregating $F_{2:3}$ families, there was a range of 1 to 14 chlorophyll-deficient mutants. The LR64 F_1 hybrid cotyledon colour was red. When F_2 mother plants were seeded, the cotyledon colour was not recorded. However, an additional 402 F_2 seeds were available for which the seed coat was partially removed by scarification so the colour of the cotyledon could be visually assessed. Of the 104 LR64 F_2 individuals evaluated for disease resistance, at least 60 and as many as 120 F_3 seeds per family were scarified and classified as non-segregating for red cotyledon colour or classified as segregating if the presence of yellow cotyledons existed. Families possessing any seeds with yellow cotyledons were characterized as segregating.

Segregation for cotyledon colour in LR64 F_2 and $F_{2:3}$ families was tested using Chi-square analysis for expected ratios based on duplicate dominant epitasis (F_2: 15 Red : 1 Yellow; $F_{2:3}$: 7 Red : 8 Segregating). Due to lethality of yellow cotyledon F_2s, $F_{2:3}$ expected ratios were adjusted. Fit to expected F_2 and $F_{2:3}$ segregation ratios for albino, *chlorina* and *xantha* mutants were tested using Chi-square analysis. Expected ratios were calculated based the assumption the original LR59-81 was heterozygous

for *chl* or *xan* (the possible genes conferring chlorophyll deficiency) or homozygous for a single, dominant gene controlling chlorophyll production. Expectations for $F_{2:3}$ families were also calculated for *chlorina* and *xantha* based on cotyledon colour being controlled by duplicate dominant epistasis and tested for fit with chi-square analysis. Spearman correlation of proportion of the chlorophyll deficient mutants and yellow cotyledon $F_{2:3}$ families was also calculated.

7.3 Results and Discussion

In the LR 64 population which was derived from the cross LR59-81 (assumed red cotyledon) x 'CDC Redberry' (red cotyledon), there was segregation for cotyledon colour in both the F_2 and $F_{2:3}$ generations suggesting there were different genes controlling red cotyledon colour in each of the parents. The segregation ratio of the F_2 individuals and $F_{2:3}$ families suggested that the proposed two red cotyledon parents have a different single dominant gene controlling cotyledon colour as the segregation ratio for duplicate dominant epitasis fits the data (Table 7.1). Ladizinsky et al. (1985) suggested a minor translocation could be responsible for a 15: 1 ratio for a single gene controlling chlorophyll production in a *L. ervoides* x *L. culinaris* interspecific population. Based on the results of these experiments, an alternative theory to independent genes controlling cotyledon colour could be disruption of the synteny of the ancestral gene due to chromosomal rearrangements during speciation. If the gamete contributed by LR59-81 was yellow, these results would suggest the populations is highly skewed towards the 'CDC Redberry' red cotyledon colour allele which, in an un-distorted population, would be expected to segregate as 3 Red : 1 Yellow ratio in the F_2 generation. Regardless the original allele in LR59-81, based on these results, it is evident that genetic control of cotyledon colour was not the expected single dominant gene that has previously been documented in *L. culinaris*.

Table 7.1. Cotyledon colour of F_2 individuals and $F_{2:3}$ families from LR64 classified for red, yellow or segregating cotyledon colour and tested for control of the trait by duplicate dominant epistasis.

Cotyledon Colour	F_2		$F_{2:3}$	
	Expected (n)	Observed (n)	Expected (n)	Observed (n)
Red	377	384	45	53
Yellow	25	18		n/a †
Segregating ‡			51	43
Total	**402**		**96**	
χ^2 §	1.59		2.28	
p-Value ¶	0.21		0.13	

† Chlorophyll-deficient mutants failed to produce $F_{2:3}$ families presumably due to lethality of the recessive allele

‡ Segregating for yellow and red cotyledon colour

§ X^2 value for test of a observed ratios of red and yellow cotyledons or red and segregating families to expected ratios for duplicate dominant epistasis

¶ Probability of a greater χ^2 value under the null hypothesis of ratio tested with Yates correction for continuity

Note: F_2 seed was from the same LR64 F_1 plant, but not the same seed as that used to derive the $F_{2:3}$ families.

As described in section 6.2.1, six highly resistant LR59 lines from the interspecific RIL population (data not shown) were originally selected for crossing and line LR59-81 proved to be the most genetically compatible when crossed with *L. culinaris* (approximately 2% crossing efficiency compared to 0% for the other five lines). At the F_{10} generation, LR59-81 seed derived from a single red cotyledon plant still segregated for approximately 85% red and 15% yellow cotyledon colour. The yellow cotyledon seeds produced chlorophyll deficient, infertile, non-viable mutant

plants (data not shown) resembling the *xantha* phenotype described by Vandenberg and Slinkard (1987). A comparison over three generation of selfing LR59-81 suggested the proportion of yellow cotyledon seeds may be decreasing (data not shown), but needs to be confirmed over a larger sample size. Interestingly, LR67 (LR59-81 x 'Eston') F_2 and $F_{2:3}$ populations were not only more uniform with normal chlorophyll production, but were also true breeding for cotyledon colour (all yellow) suggesting the allele for cotyledon colour produced by LR59-81 in the creation of LR67 was yellow.

In the LR64 population, segregation for both cotyledon colour and chlorophyll deficient mutants was observed in the F_2 population and in the $F_{2:3}$ families (Tables 7.1 and 7.2). Both generations seemed to segregate in to 15 normal : 1 albino seedlings as described in the *L. culinaris* x *L. ervoides* interspecific population by Ladizinsky et al. (1985). The F_2 segregation ratio of normal to chlorophyll-deficient mutants did not conform to those described by Vandenberg and Slinkard (1987 and 1989) for *xantha* or *chlorina* mutants (Table 7.2). The observed ratio of normal to segregating $F_{2:3}$ families was consistent with that expected for *chlorina* mutants, however when tested for segregation with the segregation for cotyledon colour, the model did not fit suggesting genes conferring chlorophyll-deficiency and cotyledon colour may be linked. Expected ratios for *xantha* mutants were rejected, even when adjusted for digenic control of red cotyledon colour in LR64. However, the proportions of the chlorophyll deficient mutants and yellow cotyledon in 48 $F_{2:3}$ families were significantly correlated (0.65, $p < 0.01$) suggesting a relationship between the two traits may exist.

Table 7.2. LR64 F_2 individuals and $F_{2:3}$ families from LR64 classified for red, yellow or segregating cotyledon colour and normal or chlorophyll deficient plants and tested for fit to models for *chlorina*, *xantha* and albino phenotypes.

		Normal F_2 plants						Chlorophyll-Deficient F_2 plants
$F_{2:3}$ Family Plant Types	**$F_{2:3}$ Family Cotyledon Colour**	**Observed n**	**Albino Expected n**	***Chlorina* Expected n †**	***Xantha* Expected n †**	**Red Cotyledon Colour controlled by Duplicate Dominant Epitasis *Chlorina* Expected n ‡**	**Red Cotyledon Colour controlled by Duplicate Dominant Epitasis *Xantha* Expected n ‡**	**Observed n**
Normal	Red	17			4	7	7	
	Segregating §	2	22	16	7	8	8	
	Yellow	0			4	1	1	
Segregating ¶	Red	9				18		
	Segregating	20	26	32	22	12	30	
	Yellow	0			11	2	2	
Total		**48**						
Chi-Square	X^2 #		078	0.70	55.14	26.75	21.72	
	***p*-Value ††**		0.38	0.40	<0.01	<0.01	<0.01	
$F_{2:3}$ Family Plant Types	**$F_{2:3}$ Family Cotyledon Colour**							
Unknown	Segregating	21						3
Unknown	Red	27						4
Total		**96**						**7**
	Ratio	96 Normal : 7 Chlorophyll-deficient						
F_2 Chi-Square		**X^2 ‡‡**	***p*-Value ††**					
	Albino	0.09	0.76					
	Chlorina or *Xantha*	17.49	<0.01					

† Expected for albino (Ladizinsky et al., 1985), *chlorina* (Vandenberg and Slinkard, 1989) or *xantha* (Vandenberg and Slinkard, 1987) chlorophyll-deficient mutants

‡ Expected for chlorine or *xantha* chlorophyll deficient mutants with red cotyledon colour being conferred by duplicate dominant epistasis

§ Segregating for yellow and red cotyledon colour

¶ Segregating for chlorophyll deficient mutants and normal plant types

X^2 value for test of a ratio of observed $F_{2:3}$ family plant types and cotyledon colour to expected ratio for *chlorina* and *xantha*

†† Probability of a greater χ^2 value under the null hypothesis of ratio tested with Yates correction for continuity

‡‡ X^2 value for test of a ratio of F_2 expected ratio of normal to chlorophyll-mutant plants for albino, *chlorina* and *xantha*

Zamir and Tadmor (1986) reported segregation distortion in 44% of loci in interspecific populations between *L. ervoides* and *L. culinaris*, however they did not find skewness of favoured alleles towards either species. Tadmor et al. (1987) found with simple linkage maps that single loci were independently distorted in an interspecific *Lens* cross suggesting only portions of, not entire chromosomes, are distorted. Thus, non-distorted cotyledon colour segregation is not necessarily an indication of lack of distortion for other traits.

7.4 Conclusions

Segregation of red to yellow cotyledons in the LR64 F_2s and $F_{2:3}$ families suggested that two independent dominant genes control red cotyledon colour, unlike the single gene control previously reported for *L. culinaris* intraspecific crosses. The segregation of chlorophyll mutants in this population resembled that observed previously for a *L. culinaris* x *L. ervoides* population, suggesting more than one cross into *L. culinaris* background may be necessary to negate incompatibilities between the genomes when introgressing alleles from the wild species. Segregation did not fit the expected ratio for *xantha* phenotypes despite the phenotypic similarities of the interspecific-derived parent LR59-81. The *xantha* phenotype described by Vandenberg and Slinkard (1987) was observed in a cross between *L. culinaris* and *L. orientalis* which are both considered to be in the primary gene pool, suggesting genetic control of this phenotype may be even more complex when introgression from the secondary gene pool has occurred. In the previous chapter, it was postulated that

segregation distortion in ratios of resistant to susceptible plants in LR64 and LR67 introgression populations were responsible for the unanticipated results on the model of genetic control for resistance. These results of this chapter support the theory that the LR64 population is not following typical Mendialian segregation for traits that have been previously characterized in lentil. Furthermore, it can be speculated that the introgression population LR64 is maintaining irregular chromosomal arrangements due to the ancient reciprocal translocation between the species.

CHAPTER 8

GENERAL DISCUSSION

Results from this project represent a small piece of the unravelling story of anthracnose in lentil. The disease is present in lentil growing regions world-wide; however is only a major disease in Saskatchewan. Thus, local research will be foundational in understanding this relatively uncharacterized pathosystem. The overall hypothesis of this project was that resistance derived from *Lens ervoides* accession L-01-827A would provide unprecedented levels of resistance to *Colletotrichum truncatum* and studies performed as part of the project addressed three key problematic issues: i) Lack of resistance to *Colletotrichum truncatum* race Ct0 in *Lens culinaris*; ii) Unreliability of single plant phenotyping of resistance, and iii) Resistance deterioration between the juvenile phase (JP) and adult phases (AP) in the line 'CDC Redberry'.

The introgression efforts made in this project will provide unmatched levels of disease control to the more the virulent race Ct0 and resistant individuals identified in the introgression populations developed have already been used in the breeding program a resistant parents. The multi-year field evaluation confirmed the unprecedented disease control that can be obtained using *L. ervoides*-derived resistance gene(s) and provides insight into appropriate screening methodology based on moderate heritability of the trait. Results from screening two subsequent generations of introgression populations showed resistance from *L. ervoides* to both races of *C. truncatum* was due to the same gene(s) or from the same linkage block and resistance was conferred by major genes. Major genes, such as those for disease resistance, have been the primary focus of most wild trait introgression efforts, however wild relatives are potentially a rich source of genes for other important traits,

including quantitative traits (Hajjar and Hodgkin, 2007; Tanksley and McCouch, 1997). It is expected that as resistance genes from L-01-827A are introgressed, other beneficial genes will also be incorporated that will contribute to increasing lentil yields. Introgressed material was first grown in yield trials in the summer of 2009 and showed potential for higher yield than checks across a number of pedigrees (Vandenberg, personal communication). However, it is suspected that meiotic abnormalities may have existed when the interspecific line was crossed with *L. culinaris* as segregation ratios of resistant to susceptible lines were highly skewed towards segregating and susceptible families. It was shown that segregation for albino mutants was similar to what had been previously observed in a *L. culinaris* x *L. ervoides* interspecific population, supporting the hypothesis of continued effects of interspecific hybridization in introgression populations.

Single plant phenotyping of resistance to *C. truncatum* in lentil is unreliable and detached leaf assays are not considered a viable option for characterizing anthracnose resistance in lentil. Thus, the feasibility of using clonal propagation of individual plants to generate replicated ratings was developed, evaluated and showed consistency in segregation ratios between cutting and seedling-derived plants of the same population. The method was utilized for testing of genetic control of segregating populations and the combination of resistance classifications of F_2 cutting-derived plants and $F_{2:3}$ seedling-derived plants proved to be the most powerful in resolving convoluted results.

Juvenile phase resistance compared to AP susceptibility was defined in 'CDC Redberry' in this project and is the first time declining resistance with increased age has been reported in this pathosystem. When inoculated with race Ct0, the partially resistant line showed young seeding susceptibility, followed by acquisition of resistance that decreased as the plants proceeded through the AP. Resistance decline was also observed with both races of *C. truncatum* with less disease caused by race Ct1. There were significant differences between the JP and AP on more than a third of the F_2s tested in the segregating population supporting different resistance gene action based on growth phase. The results of the experiments characterizing the decline in resistance resulted in more effective experimental design in population phenotyping

and eventually helped interpret the seemingly confusing results observed between the two different populations. It is possible the accumulation of minor JP resistance genes is the result of screening for resistance at the North Seed Farm nursery and cyclical recurrent selection. Based on the results of studies on JP and AP resistance, appropriate screening techniques such as adjusting the age of the plants at inoculation will be employed to select for resistance within the breeding program.

8.1 Ongoing Research

8.1.1 Anthracnose resistance

The accession PI72815 of *L. ervoides* and an accession of *L. lamottei* offer additional sources of interspecific-derived resistance to *C. truncatum*. Preliminary results on disease screening of LR26 ('Eston' / PI72815) recombinant inbred lines (RILs) suggest a similar mode of inheritance to what was observed in the raw LR59 ('Eston' / L-01-827A) interspecific population (Tullu, personal communication). It is currently unknown if resistance gene(s) from the two *L. ervoides* parents (L-01-827A and PI72815) are allelic or different genes. Linkage maps of the two interspecific populations (LR59 and LR26) are being developed (as described in section 8.1.2) and will provide insight into similarity of regions on the genome with *C. truncatum* resistance gene(s) and allow formation of hypotheses for further testing. An intraspecific *L. ervoides* population has been developed from the two *L. ervoides* lines (called LR66) which will allow for allelism testing in the wild background. It is also proposed that inheritance could also be analyzed by developing a population with LR59-81 and a highly resistant LR26 line. Since the generation and phenotypic characterization of an interspecific population between *L. lamottei* and *L. culinaris* (Fiala, 2006), very little work has been done to introgress this source of resistance into a cultivated background. Based on results of ongoing research by the Pulse Crop Pathology Group (as described in section 2.3), a deployment strategy for *C. truncatum* resistance genes will be determined. Resistance derived from *L. lamottei* may be a integral part of this strategy, thus future work on introgressing these genes may re-commence in the near future.

8.1.2 Linkage mapping within *Lens*

Lentil is an orphan crop when it comes to knowledge of molecular genetics and genomics (Ford et al., 2009). Recently, several projects have been initiated to develop expressed sequence tags for lentil with a long-term goal of providing the breeding program with tools to develop improved varieties more efficiently. However, these sequences have yet to be explored as molecular markers and placed on the lentil linkage map. Equally important as generating polymorphism rich linkage maps within a given species is anchoring maps to different populations within the same species for comparison and to maps of closely related species. The latter allows one to exploit macro-synteny relationships with relatives of reference genomes, for example the closely related *Medicago truncatula* genome.

A legume genomics group at UC-Davis has a collection of more than 1400 conserved orthologous sequence (COS) markers for legumes, based on low copy genes identified in reference legume genomes *M. truncatula*, *Lotus japonicus*, and *Glycine max*. By analyzing low copy genes, the evolutionary history of the Papilionoid subfamily of legumes is being studied. Markers developed with this technique have been shown to be useful for comparative mapping in various legume species (Choi et al., 2006; Choi et al., 2004a and 2004b). The full set of COS markers is currently being mapped in pigeonpea, chickpea, common bean, cowpea, lupin and both diploid and tetraploid peanut.

In lentil, the populations LR59, LR26, LR64, LR67 and LR66 are currently being assessed for single nucleotide polymorphisms (SNPs) within the legume COSs using an Illumina® Golden Gate Assay. Between *L. culinaris* lines ('Eston' and 'CDC Redberry') and *L. ervoides* lines (L-01-817A and PI72815), 676 SNPs were found which will be mapped in interspecific LR59 and LR26 populations. It is expected approximately half these SNPs will be useful for mapping LR64 and LR67. The phenotypic data that resulted from this project will allow placement of *C. truncatum* resistance genes as well as genes for cotyledon colour and albino plants on the linkage map. Furthermore, an intraspecific F_2 *L. ervoides* map will be generated for LR66 (L-01-817A / PI72815) based on 522 SNPs.

Transfer of genes of interest from wild relatives can often be accompanied by linkage drag of deleterious genes or traits limiting agronomic performance. Reducing linkage drag by eliminating the undesirable segments of the wild genome is done through repeated backcrossing to the adapted crop species while selecting for the gene(s) of interest. Selection for key markers associated with discreet segments of the genome can speed up the recovery of the adapted background (Fritch and Melchinger, 2001). Thus establishing a map within *L. ervoides* and interspecific populations is imperative for utilizing genes in wild accessions. These highly conserved, gene-based COS markers will be useful in tracking introgression of genes from *L. ervoides* throughout interspecific transfer to *L. culinaris*. Markers spanning the genome will be useful in identifying and eliminating deleterious and non-favourable genomic segments from the wild species that can be identified and excluded more effectively within the existing cycle of the breeding program.

It is hoped the linkage maps of LR64 and LR67 will resolve how many regions in the genome are actually involved in providing resistance to both races of *C. truncatum* and if JP resistance genes are independent of those derived from L-01-827A. The hypothesis of segregation distortion will be tested for regions with resistance genes as well as across the genome using co

mparative mapping of both intra and interspecfic populations. The resulting maps will anchor *L. culinaris* maps to other populations and other species, especially the model legume *M. truncatula*. Also being developed for legumes is comprehensive maps of resistance gene clusters based on homologs of *M. truncatula* NBS-LRR genes (Zhu et al., 2002) and comparative mapping of resistance genes with *M. truncatula* will provide another useful tool for gene discovery.

8.2 Anthracnose Rating Scale and Confirmation of Resistance Testing on Cutting-Derived Plants

It is possible that different resistance genes could be expressed in different plant parts or expressed with different phenotypes, thus quantitative measurements were taken to record three different disease parameters. The rating for percentage of stem lesions (SL) represents the coverage of the main stem with lesions, regardless the

penetration of the lesions. The proportion of the leaves covered with lesions along with the amount of defoliation due to disease is represented by the percentage of leaf lesions (LL). This parameter was not recorded on AP plants where defoliation due to disease could not be differentiated from natural senescence observed on the un-inoculated checks. The amount of penetrating lesions on stems is represented by the percentage shoot die back (SDB).

Correlation analysis of SL, LL and SDB raw ratings across all F_2 and $F_{2:3}$ experiments for both LR64 and LR67 were highly significant ($r \geq 0.80$, $p < 0.01$) suggesting the same genes may control resistance in the different organs and resistance to deep penetration of stem tissue by the pathogen. However, frequency distributions of LL were often more continuous than SL and SDB, suggesting genetic control or expression of leaf resistance may be different than that of stems. Similarly, Chongo and Bernier (2000b) found high correlations between percent sporulating stem lesions, disease incidence and stem lesion size across six lines when inoculated with *C. truncatum* .

By quantifying the percentage of disease on the three parameters, the phenotypic platform is available for identification of quantitative trait loci (QTL) for each resistance trait based on the COS markers. This information will be valuable for resolving if different resistance genes exist for the different parameters and how these genes are distributed across the genome. Geffroy et al. (2000) rated stems, leaves and petioles on bean RILs and found some evidence of specialized QTLs acting in specific organs. Stem resistance QTLs were found to be more significant than leaf QTLs which mapped to the same location, or in one instance no leaf resistance QTL was detected where a significant stem resistance QTL existed.

When comparing ratings of cuttings and seedlings of LR64 F_2s, it was reflected the SDB parameter results should be interpreted with caution (Chapter 4). There were higher numbers of shoots counted on cutting-derived plants versus seedling-derived plants. When results on disease screening of the two treatments were compared, there were minor discrepancies in significant differences, frequency distributions and results on chi-square analysis for models reflecting genetic control. However, ratings on cutting-derived F_2s compared with seedling-derived $F_{2:3}$ families had consistent results

on both SL and SDB parameters (Tables 6.2, 6.5, 6.8 and 6.9) with the differentiation of susceptible and resistant classifications being clearer on seedling-derived plants inoculated under controlled conditions. The combination of resistance classifications of F_2 cutting-derived plants and $F_{2:3}$ seedling-derived plants proved to be the most powerful in resolving the convoluted results from these studies (Table 6.10). Furthermore, there was significant correlation of means from both generations, especially when both populations were inoculated exclusively under controlled conditions (Figure 6.2).

8.3 Suggestions for the CDC Lentil Breeding Program and Future Research

8.3.1 Field studies and evaluation of anthracnose resistance

For the field study of the LR59 RILs, it would have been preferential to screen more lines, however variable seed set and fertility limited the number of lines available. Alternatively, RILs developed from LR64 and LR67 could be used to re-assess the heritability of resistance derived from L-01-827A as seed will soon be available for these populations. In order to more accurately reflect selection for resistance within the breeding program, it may be preferential to focus resources on multi-season evaluation at the North Seed Farm to ensure disease infection as such low levels of disease were observed at the Preston location, especially in 2007. The heritability value obtained for the LR59 population is a good indication that selection based on F_2 individuals should be confirmed at another point during the breeding cycle, especially when the F_2 nursery season is not conducive to anthracnose infection. Currently, coop trial entries are screened for race Ct1 resistance in replicated experiments under controlled conditions. As breeding lines with *L. ervoides*-derived resistance genes near the latter portion of the breeding cycle, indoor screening with race Ct0 may be useful in selecting potential varieties between various families and selecting breeder seed lines within the selected families.

The second location for the field trial with delayed seeding date (Preston), had much lower disease levels in the field study. Since that location has a history that would tend to lower soil-inoculum levels of anthracnose, it is hard to say which factor

contributed more to lower disease levels. Based on these results and those of the plant age study, it would be interesting to study the effect of planting date under high disease pressure. Such an experiment could be conducted at the North Seed Farm over a few varying seasons using as few as six different lines, such as 'CDC Redberry', 'Eston', VIR421, 'Indianhead', 'CDC Robin' and LR59-81 using flanking plots with the highly susceptible 'Pardina'. Instead of using single-row plots to assess disease, multi-rowed microplots or bordered-plots would be useful as it was found larger plant types overgrew smaller ones in the study from this project. Results would be insightful into disease mitigation strategies given different environmental conditions as an alternative to fungicidal application.

8.3.2 Phase-dependent resistance

The JP resistance described in this project presents an interesting area of potential study. Results presented in Chapter 5 suggested under specific conditions, young seedlings of *L. culinaris* may be highly susceptible to race Ct0. Further testing of various ages of plants within the JP is necessary for confirming young seeding susceptibility. Underlying mechanisms of seedling susceptibility and JP resistance could be elucidated using a variety of techniques including cytology, gene expression studies and identification of QTLs for the trait. Using microarray analysis, candidate genes involved in Asian soybean rust resistance were identified for different plant stages (Panthee et al., 2008). A similar analysis could be done on 'CDC Redberry' in seedling, JP and AP plants inoculated with *C. truncatum* using a *M. truncatula* microarray chip. This type of study may be valuable in sorting out the interaction of developmental and disease resistance pathways. The microarray chip, or candidate genes identified from this type of analysis could then be used to generate expression QTLs in LR64 RILs re-phenotyped for JP and AP resistance which would further understanding of the biological mechanisms of the trait and how to select for it.

Indoor screening of coop trial entries for resistance is typically done using four week old plants. Results from this project suggest screening of six week old plants, especially to race Ct0, may more accurately reflect overall resistance. However, the increase cost of growing plants and additional time and ease of inoculating larger

plants may warrant this practice unsuitable. Selection for AP resistance may be more suitable using marker-assisted selection if an appropriate marker is identified from the COS maps of LR59, LR64 and LR67.

8.3.3 Additional populations and potential genetic studies

The introgression populations are both segregating for many other traits not previously described. For example, it was found the *L. ervoides* parent L-01-827A showed photoperiod insensitivity and LR64 F_2s were segregating for the trait. Other variable traits include plant type, height, determinacy, number of pods per node, seed size, dehiscence, seed background colour and seed coat patterns and plant vigour. Both LR64 and LR67 populations have been developed into RILs, thus are available for phenotyping for other traits. Interestingly, LR64 RILs show a much larger range in plant phenotypes than the LR67 population, similar to what was observed in the F_2 and $F_{2:3}$ generations.

Additional crosses were made at the onset of this project that were not studied, but provide a resource of material for subsequent studies. For example, near isogenic lines of 'Eston' and a *C. truncatum* resistant 'Eston' are partially generated as several resistant F_3 LR67 plants were crossed back to 'Eston'. Having these lines available will be valuable for in-depth study of the interaction of host and pathogen in this pathosystems. Crosses between VIR421 and LR59-81 have been made and the resulting F_1 seed could be used to generate populations in which allelism of resistance genes from these two sources could be studied. A cross between VIR421 and 'Eston' has been developed into RILs (called LR65) and is also available to study this additional source of resistance gene(s).

8.4 Conclusion

Introgression of resistance genes from wild *Lens* species is proving to be a valuable source of resistance for anthracnose in lentil. Results from this project show that interspecific-derived resistance from *L. ervoides* accession L-01-817A will provide unprecedented control of the pathogen. Field-evaluation, inoculations with virulent race Ct0 and inoculations in AP plants show resistance is much higher than

any identified in the *L. culinaris* gene pool. Furthermore, resistance is due to dominant, major genes, thus should increase the ease of selection for anthracnose resistance within the breeding program. However, the results of this project also demonstrated some of the difficulties that can be encountered when introgressing resistance genes from the secondary gene pool as the populations studied were still highly distorted preventing firm conclusions on the number of genes controlling the traits evaluated.

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APPENDICIES

Appendix 1: Mixed model analysis of variance of disease ratings on lentil lines evaluated in anthracnose disease nurseries in the growing seasons of 2006 and 2007.

	Source of Variation	Num df	Den df	F-Value	*p*-Value	Estimate†
Combined	Line	29	29.6	6.52	<0.01	
	Susceptible Covariate	1	344	38.86	<0.01	
	Resistant Covariate	1	279	61.82	<0.01	
	Year					0
	Site					0
	Year x Site					0
	Block(Year x Site)					0
	Year x Line					353323
	Site x Line					0
	Year x Site x Line					711941
	Residual					533114
2006	Line	29	29.4	14.46	<0.01	
	Susceptible Covariate	1	199	12.54	<0.01	
	Resistant Covariate	1	206	23.04	<0.01	
	Site					0
	Block(Site)					0
	Site x Line					383158
	Residual					856836
2007	Line	29	29	2.85	<0.01	
	Susceptible Covariate	1	81.6	10.06	<0.01	
	Resistant Covariate	1	107	0.23	0.63	
	Site					125187
	Block(Site)					307
	Site x Line					1030608
	Residual					191417

2006 North Seed Farm	Line	29	85.3	13.32	<0.01	
	Susceptible Covariate	1	68	1.39	0.24	
	Resistant Covariate	1	39	0.60	0.44	
	Block					7311
	Residual					1425239
2006 Preston	Line	29	86	58.49	<0.01	
	Susceptible Covariate	1	86	10.54	<0.01	
	Resistant Covariate	1	86	18.71	<0.01	
	Block					0
	Residual					293518
2007 North Seed Farm	Line	29	88	56.92	<0.01	
	Susceptible Covariate	1	88	1.42	0.24	
	Resistant Covariate	1	88	0.06	0.81	
	Block					0
	Residual					268521
2007 Preston	Line	29	83	10.22	<0.01	
	Susceptible Covariate	1	59.5	8.52	<0.01	
	Resistant Covariate	1	69.8	1.15	0.29	
	Block					1212
	Residual					107128

† Estimate of the components of variance for the random effects.

Appendix 2. Mixed model analysis of variance of disease ratings on lentil $F_{2:3}$ families from the cross Yerli Kirmizi / 'Redberry' // 'Redberry' (3155S) evaluated in anthracnose disease nurseries in the growing seasons of 2006 and 2007.

		2006				2007				Combined			
Source of Variation	**Num df**	**Den df**	**F-Value**	***p*-Value**	**Estimate**	**Den df**	**F-Value**	***p*-Value**	**Estimate**	**Den df**	**F-Value**	***p*-Value**	**Estimate**
Line	3	3.02	2.32	0.25		19.7	0.98	0.42		2.9	0.58	0.67	
Susceptible Covariate	1	20.9	13.35	<0.01		1	2.29	0.37		29.4	10.34	<0.01	
Resistant Covariate	1	24.9	17.94	<0.01		25.9	7.51	0.01		25.3	34.93	<0.01	
Year													0
Site					0				7170				0
Year*Site													210741
Block(Year*Site)													58153
Block(Site)					131832				4105				
Year*Line													111141
Site*Line					177726				0				8857
Year*Site*Line													56813
Residual					164411				68544				134873

Appendix 3. Mixed model analysis of variance of disease ratings on *Lens culinaris* lines evaluated in anthracnose disease nurseries in the growing season of 2006 and 2007.

Source of Variation	Num df	2006				2007				Combined			
		Den df	F-Value	*p*-Value	Estimate	Den df	F-Value	*p*-Value	Estimate	Den df	F-Value	*p*-Value	Estimate
Line	10	10.1	14.59	<0.01		10	2.45	0.08		31.2	12.97	<0.01	
Susceptible Covariate	1	72.2	21.16	<0.01		46.6	11.59	<0.01		149	43.75	<0.01	
Resistant Covariate	1	66.3	49.16	<0.01		49.2	5.41	0.02		155	68.07	<0.01	
Year													0
Site					0				68150				10818
Year*Site													0
Block(Year*Site)													0
Block(Site)					2208				3381				
Year*Line													0
Site*Line					325246				1588846				0
Year*Site*Line													661671
Residual					175754				196847				193902

Appendix 4. Mixed model analysis of variance of disease ratings on *Lens ervoides* lines evaluated in anthracnose disease nurseries in the growing season of 2006 and 2007.

		2006				2007				Combined			
Source of Variation	**Num df**	**Den df**	**F-Value**	***p*-Value**	**Estimate**	**Den df**	**F-Value**	***p*-Value**	**Estimate**	**Den df**	**F-Value**	***p*-Value**	**Estimate**
Line	4	4.04	16.74	<0.01		4.94	4.32	0.07		4.4	12.76	0.01	
Susceptible Covariate	1	31.6	4.2	<0.05		33	4.43	0.04		53.7	5.63	0.02	
Resistant Covariate	1	32.6	0.05	0.83		28.4	0.04	0.84		42.8	0.4	0.53	
Year													0
Site					0				0				0
Year*Site													0
Block(Year*Site)													0
Block(Site)					0				0				
Year*Line													135091
Site*Line					690831				1733224				0
Year*Site*Line													1455128
Residual					1542024				201301				859189

Appendix 5. Mixed model analysis of variance of disease ratings on *Lens culinaris* x *Lens ervoides* recombinant inbred lines evaluated in anthracnose disease nurseries in the growing season of 2006 and 2007.

		2006				2007				Combined			
Source of Variation	**Num df**	**Den df**	**F-Value**	***p*-Value**	**Estimate**	**Den df**	**F-Value**	***p*-Value**	**Estimate**	**Den df**	**F-Value**	***p*-Value**	**Estimate**
Line	13	13.3	11.79	<0.01		12.9	1.4	0.28		15.5	2.1	0.08	
Susceptible Covariate	1	80.4	2.33	0.13		10.3	5.29	0.04		61	9.99	<0.01	
Resistant Covariate	1	65.5	10.68	<0.01		88.1	1.71	0.19		30.4	48.25	<0.01	
Year													0
Site					0				50034				0
Year*Site													0
Block(Year*Site)													32005
Block(Site)					49016				1762				
Year*Line													790670
Site*Line					216122				308476				117898
Year*Site*Line													132763
Residual					1099868				169709				642270

Appendix 6. The range, median and mean of number of nodes and number of nodes with flower buds on the combined F_2s from LR64, parental lines 'CDC Redberry' and LR59-81 and check line 'Eston'.

	Line	Range (n)		Median (n)		Mean (n)		Mean
		JP †	AP	JP	AP	JP	AP	JP vs. AP ‡
Nodes (n)	LR59-81	4-9	7-16	7	11	7	12	5 ± 0.2**
	Eston	3-9	8-16	7	12	6	12	6 ± 0.2**
	CDC Redberry	2-10	7-15	6	11	6	11	5 ± 0.2**
	F_2	1-10	2-17	7	12	6	12	6 ± 0.1**
Nodes with Flower Buds (n)	LR59-81	0-2	0-12	0	2	0	2	2 ± 0.3**
	Eston	0-4	0-13	0	4	0	4	4 ± 0.3**
	CDC Redberry	0-1	0-10	0	1	0	2	2 ± 0.3**
	F_2	0-7	0-16	0	3	1	4	4 ± 0.1**

† Range, median or mean of plants at juvenile phase (JP) inoculated 21 days after cuttings taken and adult phase (AP) inoculated 42 days after cuttings taken.

‡ Difference in means between JP and AP, standard error of the mean and significance level.

** Significantly different from zero at $p < 0.01$.

Appendix 7. Mixed model analysis of variance of stem and leaf lesions and shoot die back on *Lens culinaris* pooled LR64 and LR67 F_2 and F_1 individuals, parents and checks inoculated in the adult phase with race Ct0 and race Ct1 of *Colletotrichum truncatum*.

Population	Source of Variation	Num df	Stem Lesions†				Leaf Lesions‡				Shoot Die Back§			
			Den df	F-Value	*p*-Value	Estimate	Den df	F-Value	*p*-Value	Estimate	Den df	F-Value	*p*-Value	Estimate
LR64	Race	1	2200.00	5.16	0.02		19.8	7.32	0.01		12.8	17.05	<0.01	
	Line	4	15.5	290.25	<0.01		9.6	46.09	<0.01		12.6	12.6	<0.01	
	Race x Line	4	2197.0	0.75	0.56		7.9	2.76	0.11		13.67	13.67	<0.01	
	Expt					0.02				39.83				0.07
	Expt (Bloc)					0.01				0.00				0.00
	Expt x Race					0.00				0.00				0.00
	Expt (Bloc x Race)					0.00				3.81				0.01
	Expt x Line					0.01				34.01				0.21
	Expt x Race x Line					0.00				6.07				0.00
	Residual					0.90								
	LR59-81									384.06				3.93
	Eston									17.39				0.08
	F_1									106.12				3.80
	F_2									735.28				16.50
	CDC Redberry									226.75				3.87
LR67	Race	1	4.6	16.25	0.01		19.6	2.48	0.13		61.1	1.9	0.17	
	Line	4	10.9	234.38	<0.01		9.57	29.04	<0.01		12.6	14.22	<0.01	
	Race x Line	4	17.4	8.34	<0.01		206	1.22	0.3		209.0	1.06	0.38	
	Expt					0.0018				0.00000				0.00000
	Expt (Bloc)					0.0002				0.00000				0.00000
	Expt x Race					0.0003				0.00000				0.00000
	Expt (Bloc x Race)					0.0002				0.00000				0.00000
	Expt x Line					0.0013				0.00008				0.00000
	Expt x Race x Line					0.0000				0.00000				0.00000
	Residual													
	LR59-81					0.0058				0.00239				0.00006
	Eston					0.0077				0.00004				0.00013
	F_1					0.0158				0.00050				0.00035
	F_2					0.0843				0.00010				0.00023
	CDC Redberry					0.0162								0.00041

† LR67 data Log (base 100) transformed and grouped by line to adjust heterogeneity of race and line and LR64 population cubic transformed to adjust heterogeneity of repeats and race;

‡ LR67 population reciprocal transformed and grouped by line to adjust heterogeneity of race and line and LR64 population grouped by line to adjust heterogeneity of line;

§ LR67 population reciprocal transformed and grouped by line to adjust heterogeneity of race and line and LR64 population square root transformed with $3/8^{th}$ added to values and grouped by line to adjust for heterogeneity of race and line.

Appendix 8. Mixed model analysis of variance of stem lesions and shoot die back on *Lens culinaris* pooled F_2s from LR64, parents and checks inoculated with *Colletotrichum truncatum* race Ct0 at juvenile and adult phases.

Source of Variation	Num df	Stem Lesions † Den df	F-Value	*p*-Value	Estimate	Shoot Die Back Den df	F-Value	*p*-Value	Estimate
Phase	1	2.82	90.35	<0.01		16.5	27.01	<0.01	
Line	3	8.73	318.21	<0.01		11.4	119.35	<0.01	
Phase x Line	3	6.89	53.00	<0.01		10.4	10.22	<0.01	
Expt					0.0055				42.84
Expt (Bloc)					0.0003				0.00
Expt x Phase					0.0002				0.00
Expt (Bloc x Phase)					0.0019				28.49
Expt x Line					0.0192				19.91
Expt x Phase x Line					0.0009				20.16
Residual					0.1828				1336.16
LR59-81					0.1828				
Eston					0.0556				
F_2					1.4113				
CDC Redberry					0.4255				

† Values cubic transformed and grouped by line to adjust heterogeneity of phase and line.

Appendix 9. Mixed model analysis of variance of stem and leaf lesions and shoot die back on LR64 and LR67 *Lens culinaris* F_2 individuals inoculated in the adult phase with race Ct0 and race Ct1 of *Colletotrichum truncatum*.

Population	Source of Variation	Num df	Stem Lesions †				Leaf Lesions ‡				Shoot Die Back §			
			Den df	F-Value	*p*-Value	Estimate	Den df	F-Value	*p*-Value	Estimate	Den df	F-Value	*p*-Value	Estimate
LR64	Race	1	7.2	12.24	<0.01		7.0	2.07	0.19		7.8	16.89	<0.01	
	Line	103	281.0	28.00	<0.01		287.0	15.97	<0.01		283.0	13.26	<0.01	
	Race x Line	102	1018.0	1.09	0.26		1024.0	1.22	0.08		275.0	1.20	0.13	
	Expt					0.031				0.599				0.00
	Expt (Bloc)					0.017				0.402				0.09
	Expt x Race					0.000				0.000				0.00
	Expt (Bloc x Race)					0.006				0.472				0.04
	Expt x Line					0.030				0.337				0.03
	Expt x Race x Line					0.000				0.000				0.20
	Residual					0.360				4.626				2.74
LR67	Race	1	7.3	19.02	<0.01		3.1	5.24	0.10		1029.0	0.36	0.55	
	Line	113	306.0	48.10	<0.01		303.0	16.44	<0.01		271.0	3.60	<0.01	
	Race x Line	111	1043.0	1.10	0.23		1037.0	1.25	<0.05		1015.0	0.98	0.53	
	Expt					0.020				0.0034				0.0000
	Expt (Bloc)					0.004				0.0040				0.0000
	Expt x Race					0.000				0.0004				0.0000
	Expt (Bloc x Race)					0.004				0.0011				0.0000
	Expt x Line					0.002				0.0030				0.0000
	Expt x Race x Line					0.000				0.0000				0.0000
	Residual					0.068				0.0333				0.0002

† LR67 population Log transformed to adjust heterogeneity of races LR64 population Cubic transformed to adjust heterogeneity of repetitions and blocks;

‡ LR67 population Log transformed to adjust heterogeneity of races LR64 population Square transformed to adjust heterogeneity of repetitions;

§ LR67 population Reciprocal transformed to adjust heterogeneity of repeats and LR64 population Arcsine transformed to adjust heterogeneity of races.

Appendix 10. Mixed model analysis of variance of stem and leaf lesions and percentage shoot die back on *Lens culinaris* $F_{2:3}$ families from LR64, parents and checks inoculated in the juvenile phase with *Colletotrichum truncatum* race Ct0.

			Stem Lesions				Leaf Lesions				Shoot Die Back			
	Source of Variation	Num df	Den df	F-Value	*p*-Value	Estimate	Den df	F-Value	*p*-Value	Estimate	Den df	F-Value	*p*-Value	Estimate
Overall Means	Line	3	2251	306.66	<0.01		2252	243.40	<0.01		2252	218.42	<0.01	
	Rep					0				29.42				0
	Rep(Tray Inoc)					239.09				211.83				223.77
	Residual					689.27				584.28				980.28
$F_{2:3}$ Family Means	Line	47	170	19.81	<0.01		221	13.02	<0.01		169	16.97	<0.01	
	Rep					1.79				39.39				13.69
	Rep(Tray Inoc)					37.38				54.36				48.76
	Residual					533.07				495.31				727.40
Overall Variances	Line	3	225	16.09	<0.01		228	5.07	<0.01		226	19.98	<0.01	
	Rep					256609				9399.57				1267315
	Rep(Tray Inoc)					0				0				0
	Residual					175761				187368				648963
$F_{2:3}$ Family Variances	Line	47	9.31	3.28	0.03		20.6	1.76	0.08		12.4	3.43	0.01	
	Rep					0				14342				21907
	Rep(Tray Inoc)					62624				17888				102876
	Residual					52765				69015				137494

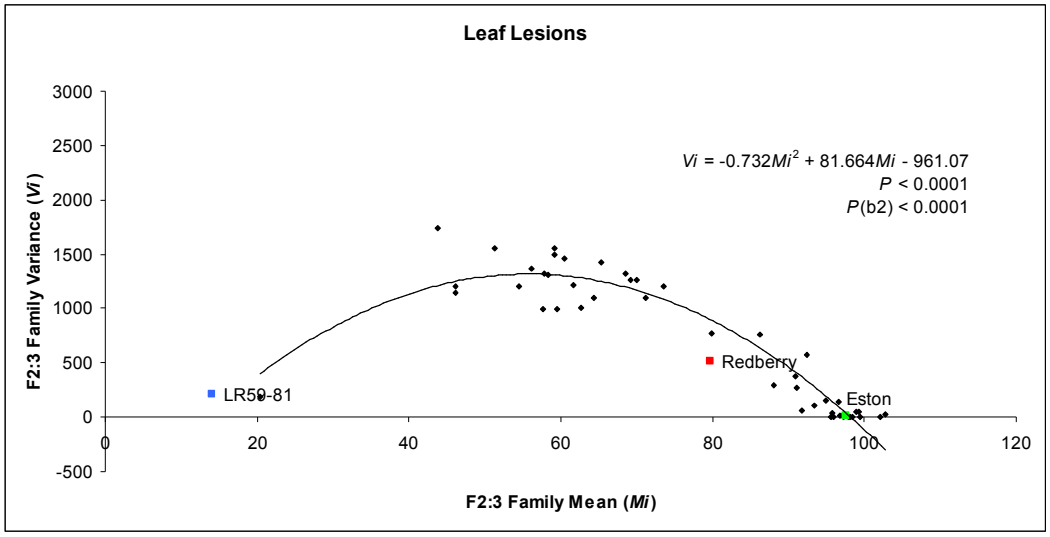
Appendix 11. Mixed model analysis of variance of stem and leaf lesions and shoot die back on *Lens culinaris* $F_{2:3}$ families from the cross LR67, parents and checks inoculated in the adult phase with race Ct0 of *Colletotrichum truncatum*.

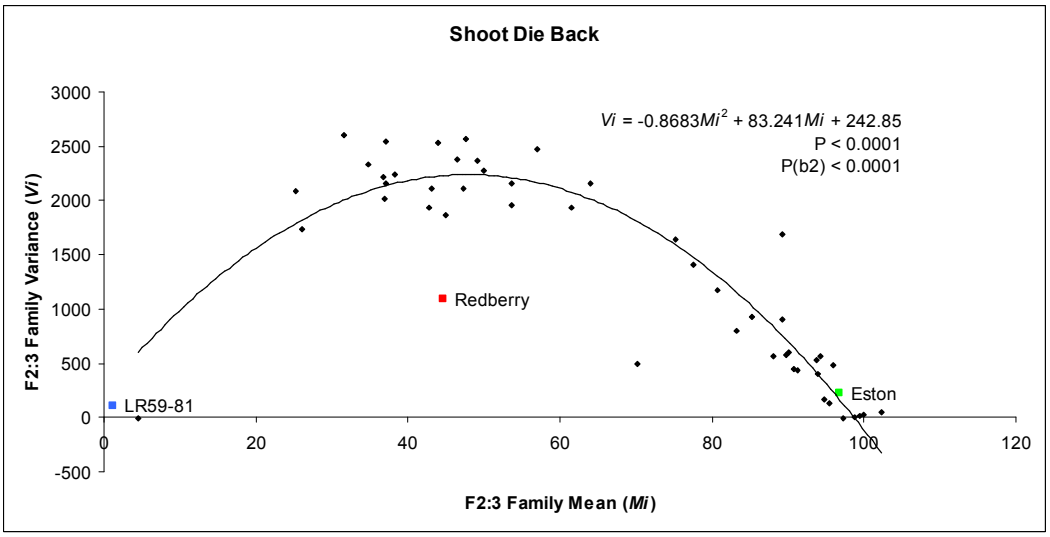
			Stem Lesions				Leaf Lesions				Shoot Die Back			
	Source of Variation	Num df	Den df	F-Value	*p*-Value	Estimate	Den df	F-Value	*p*-Value	Estimate	Den df	F-Value	*p*-Value	Estimate
Overall Means	Line	3	1968	244.95	<0.01		1968	254.51	<0.01		1967	175.68	<0.01	
	Rep					28.83				14.82				58.07
	Rep(Tray Inoc)					184.14				138.10				235.29
	Residual					985.61				752.04				1443.96
$F_{2:3}$ Family Means	Line	47	229	12.59	<0.01		244	12.1	<0.01		155	11.05	<0.01	
	Rep					41.08				26.78				79.06
	Rep(Tray Inoc)					84.65				69.72				78.75
	Residual					885.23				653.41				1302.89
Overall Variances	Line	3	188	34.42	<0.01		188	18.33	<0.01		187	30.56	<0.01	
	Rep					5563.57				9653.27				0.00
	Rep(Tray Inoc)					9137.80				7642.02				8959.09
	Residual					325207.00				294273.00				764507.00
$F_{2:3}$ Family Variances	Line	47	1	385.68	0.04		9.84	17.12	<0.01		17.8	4.93	<0.01	
	Rep					12399313.00				20500.00				46983.00
	Rep(Tray Inoc)					2239140000.00				77344.00				133142.00
	Residual					1803.89				30380.00				258975.00

Appendix 12. Mixed model analysis of variance of stems lesions as well as plot variances on *Lens culinaris* $F_{2:3}$ families from the cross LR64, parents and checks grown in an anthracnose disease nursery in 2008.

	Source of Variation	Num df	Den df	F-Value	*p*-Value	Estimate
Overall Means	Line	2	1417	156.25	<0.01	
	Rep					4.23
	Block (Rep)					16.53
	Column (Rep)					38.01
	Residual					
	Eston					786.27
	$F_{2:3}$s					946.78
	LR59-81					432.92
	CDC Redberry					872.44
$F_{2:3}$ Family Means	Family	98	3088	6.52	<0.01	
	Rep					4.05
	Block (Rep)					36.99
	Column (Rep)					26.02
	Residual					882.10
Overall Variances	Line	3	446	9.48	<0.01	
	Rep					3602.41
	Block (Rep)					1132.52
	Column (Rep)					2946.08
	Residual					161465.00
$F_{2:3}$ Family Variances	Family	98	152	1.09	0.32	
	Rep					0.00
	Block (Rep)					0.00
	Column (Rep)					536.65
	Residual					216450.00

Appendix 13. Variance with in a $F_{2:3}$ family as a quadratic function of its family mean from LR67 inoculated in the adult phase with race Ct0 of *Colletotrichum truncatum* for leaf lesions and shoot die back.





Appendix 14. Frequency distributions of stem and leaf lesions and shoot die back of *Lens culinaris* F_2s individuals from the cross LR64 with race Ct0 of *Colletotrichum truncatum*.

		SL†		LL		SDB	
		n	**%**	**n**	**%**	**n**	**%**
Disease (%)	0	4	2	4	2	17	9
	1-25	85	43	83	42	73	37
	26-50	43	22	47	24	43	22
	51-75	23	12	22	11	20	10
	76-100	41	21	40	20	43	22
Total (n)		196		196		196	
Means (%)	CDC Redberry	53.7		47.4		53.9	
	LR59-81	19.6		20.9		21.1	
	Eston	96.6		96.5		98.1	

† The number of F_2 individuals and $F_{2:3}$ families classified accordingly based on percentage of the main stem covered in lesions (SL), leaf lesions (LL) or shoot die back (SDB).

Note: Means based on four replications of cutting-derived plants inoculated with a concentration of 4 x 10^4 spores / mL three weeks after cuttings were taken and rated 21d post-inoculation. At the time of inoculation, approximately 64% of F_2 individuals, 10% of the LR59-81 plants and 0% of the 'CDC Redberry' plants were flowering.

Appendix 15. Disease response characteristics of F_3 individuals from LR64 tested for resistance in the field and to race Ct0 in the juvenile phase and from LR67 in the adult phase tested for resistance to race Ct0 of *Colletotricum truncatum.*

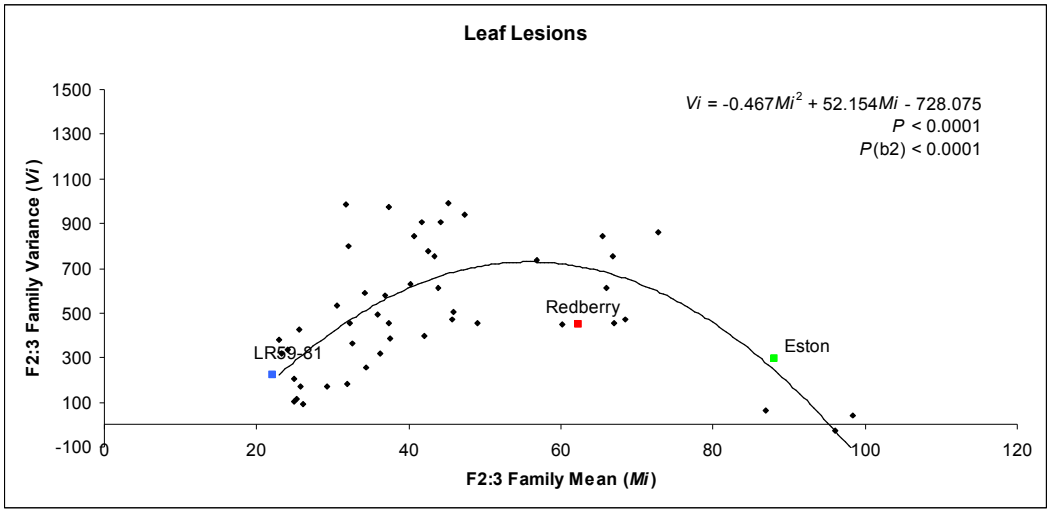
	LR64				LR67		
	Disease Nursery	Race Ct0 in Juvenile Phase			Race Ct0		
Disease response characteristic	**SL** †	**SL**	**LL**	**SDB**	**SL**	**LL**	**SDB**
P(D) – Normality test ‡	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Skewness (symmetry)	0.95	1.25	0.67	1.83	-0.87	-1.14	-0.70
Kurtosis (peakedness)	-0.52	0.11	-0.85	1.52	-1.03	-0.53	-1.43
P(|t|) – Departure from mid-parent value §	<0.0001	0.03	<0.01	<0.01	<0.0001	<0.0001	<0.0001

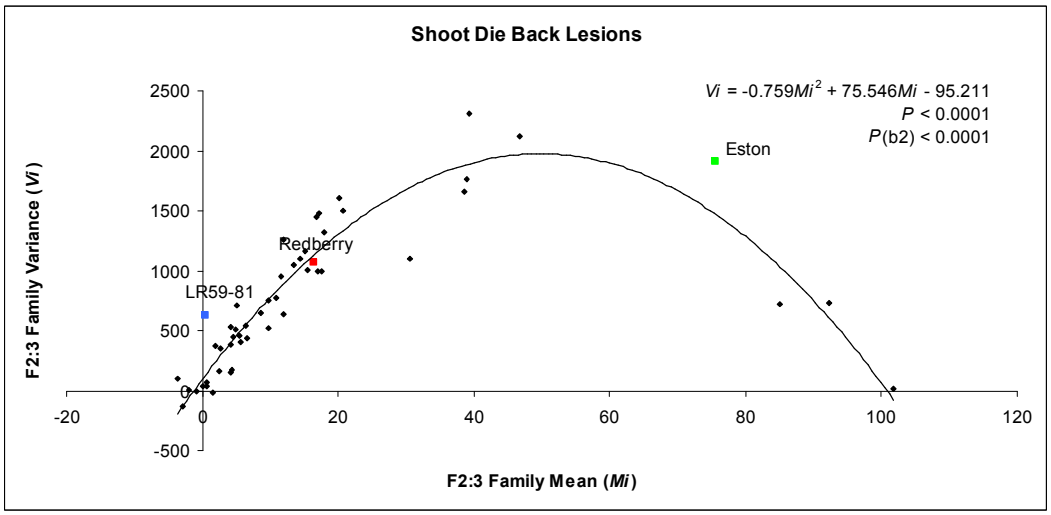
† Percentage of the main stem covered in lesions (SL), leaf lesions (LL) or shoot die back (SDB).

‡ Kolmogorov-Smirnov test for null hypothesis that the frequency distribution is normal

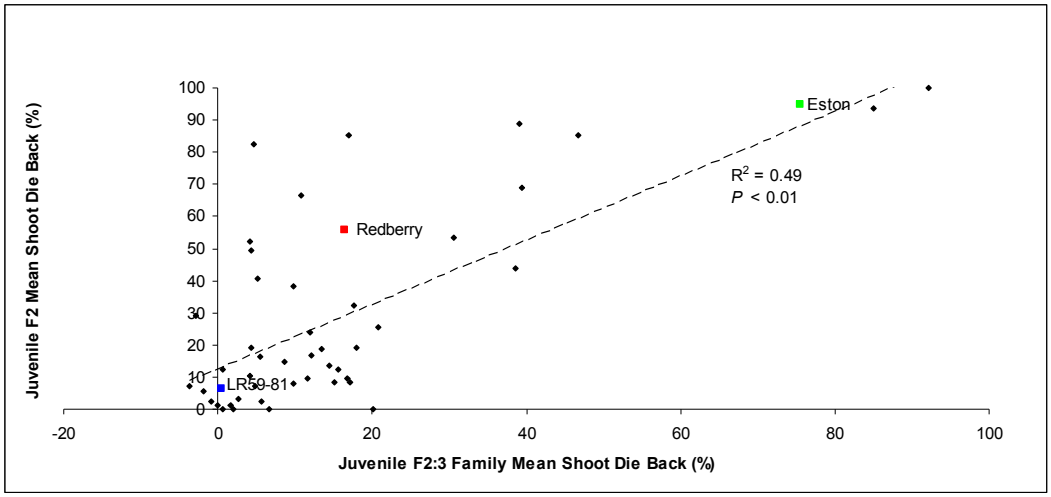
§ Null hypothesis is that there is no difference between the population mean and the mid-parental value.

Appendix 16. Leaf lesions and shoot die back variance within a $F_{2:3}$ family as a quadratic function of the family mean from LR64 inoculated at the juvenile phase with *Colletotrichum truncatum* race Ct0.

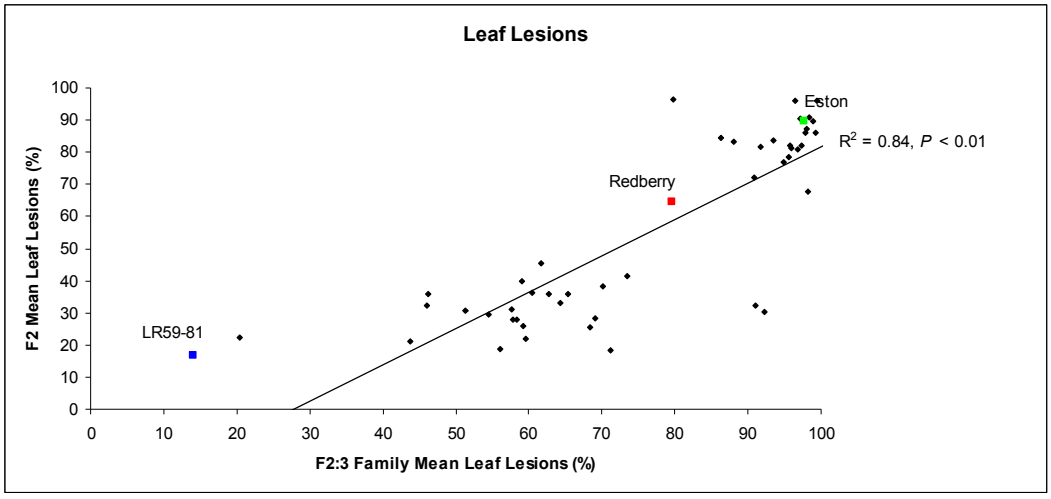


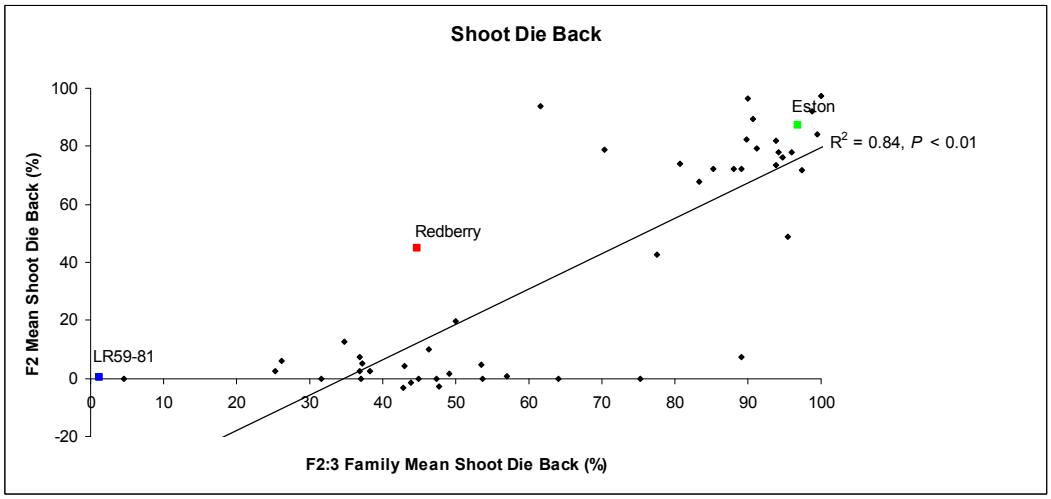


Appendix 17. Mean shoot die back of juvenile-phased LR64 F_2 individuals inoculated with *Colletotrichum truncatum* race Ct0 versus $F_{2:3}$ family means.

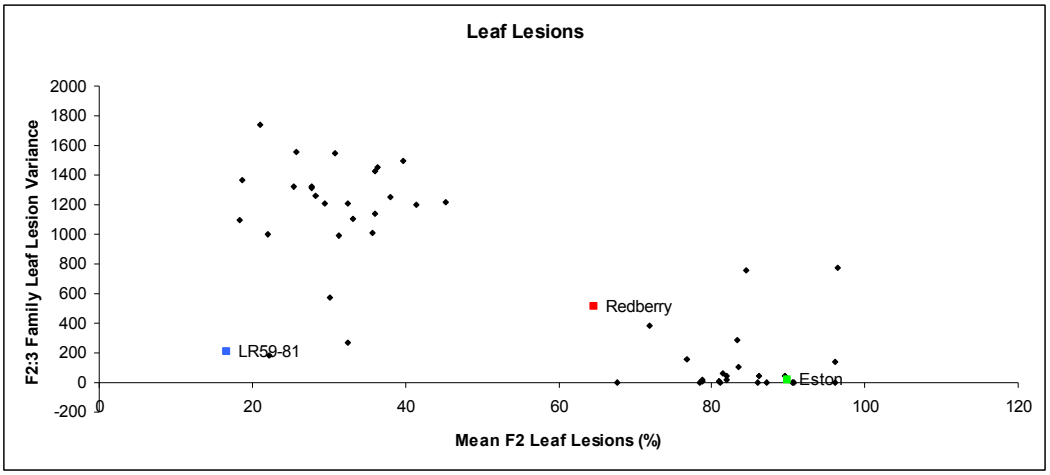


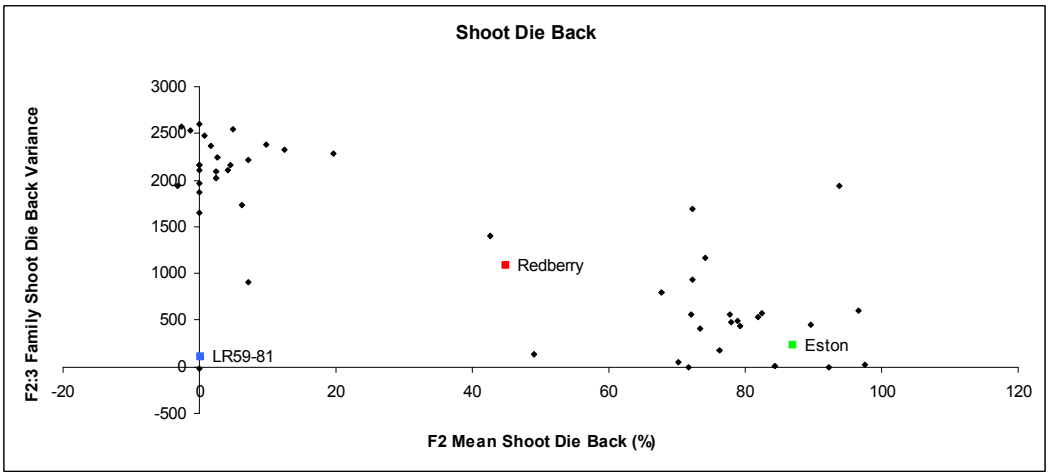
Appendix 18. Mean leaf lesions and shoot die back of LR67 F_2 individuals inoculated in the adult phase with race Ct0 of *Colletotrichum truncatum* versus $F_{2:3}$ family means.





Appendix 19. Mean leaf lesions and shoot die back of LR67 F_2 individuals inoculated in the adult phase with race Ct0 of *Colletotrichum truncatum* versus $F_{2:3}$ family variance.





Appendix 20. Chi-Square test of genetic models on F_2 individuals from LR64 inoculated with race Ct0 of *Colletotrichum truncatum* for stem and leaf lesions and shoot die back means.

	N †	196	
Stem Lesions	Ratio ‡	132:64	
	Genetic Model	χ^2 §	***p*-Value** ¶
	3:1	5.82	0.02
	15:1	245.81	<0.01
	13:3	23.20	<0.01
	9:7	9.79	<0.01
	43:21	0.002	0.96
Leaf Lesions	Ratio	134:62	
	Genetic Model	χ^2	***p*-Value**
	3:1	5.05	0.03
	15:1	236.47	<0.01
	13:3	21.48	<0.01
	9:7	10.71	<0.01
	43:21	0.12	0.73
Shoot Die Back	Ratio	133:63	
	Genetic Model	χ^2	***p*-Value**
	3:1	5.05	0.02
	15:1	236.47	<0.01
	13:3	21.48	<0.01
	9:7	10.71	<0.01
	43:21	0.04	0.85

Note: Means based on four replications of cutting-derived plants inoculated with a concentration of 4 x 10^4 spores / mL three weeks after cuttings were taken and rated 21d post-inoculation. At the time of inoculation, approximately 64% of F_2 individuals, 10% of the LR59-81 plants and 0% of the 'CDC Redberry' plants were flowering.

† Number of F_2 individuals analyzed

‡ Resistant : Susceptible with resistant individuals having ≤50% disease based on the least square mean

§ X^2 value for test of given ratio

¶ Probability of a greater χ^2 value under the null hypothesis of ratio tested with Yates correction for continuity

Appendix 21. Mean shoot die back of juvenile-phased LR64 F_2 individuals inoculated with race Ct0 of *Colletotrichum truncatum* versus $F_{2:3}$ family variance.